PAG1- a Novel Hypoxia Regulated Gene

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To my father

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Abbreviations

µg	microgram
µl	microliter
a	alanine
aa	amino acid
ADP	adenosine di-phosphate
AKT	RAC-alpha serine/threonine-protein kinase
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
BCA	bicinchoninic acid assay
bp	base pair
CpG	cytosine-phosphate-guanine
DNA	Deoxyribonucleic acid
E	embryonic day
ELK-1	member of the ETS oncogene family
ER	endoplasmatic reticulum
ERK1/2	extracellular signal-regulated protein kinases 1/2
Fok	Flavobacterium okeanokoites
HR	homologues recombination
HUVEC	human umbilical vein endothelial cells
kb	kilobases
kDa	kilodalton
LYP	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
MEF	mouse embryonal firboblast
min	minute
NAD+	Nicotinamide adenine dinucleotide phosphate (oxidated form)

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ng	nanogram
p	proline
p300	E1A binding protein p300
p53	tumor protein p53
PBS	phosphate buffered saline
PEP	proline-, glutamic acid-, serine-, and threonine-rich [PEST]-domain phosphatase
PEST	proline-, glutamic acid-, serine-, and threonine-rich-domain
pO_2	oxygen partial pressure
PR	proline rich motif
SV40	simian virus 40
SWI/SNF	SWItch/Sucrose Non Fermentable
TEL-AML1	B lineage leukemia with t(12,21) rearrangement
Tyr	tyrosine
UTR	untranslated region

Summary

An imbalance between oxygen supply and consumption leads to low oxygen conditions, also called hypoxia. This event is an essential feature in certain diseases like cancer, arteriosclerosis, stroke and inflammation. The cellular adaptation to hypoxia is mastered by the hypoxia-inducible transcription factors (HIFs). HIFs consist of a heterodimer between the constitutively expressed form HIF-β, also called aryl hydrocarbon receptor nuclear translocator (ARNT), and the tightly oxygen-controlled subunit HIF-α. In humans, three HIF-α isoforms have been described: HIF-1α, HIF-2α and HIF-3α. Under well oxygenated conditions, the prolyl-4-hydroxylases (PHD) 1-3 hydroxylate the HIF-α subunit on two prolyl residues within their oxygen- dependent degradation domain. This leads to recognition of HIF-α by the von Hippel-Lindau protein (pVHL) and subsequent proteasomal degradation. In addition, the factor inhibiting HIF (FIH) hydroxylates the HIF-α subunits at an asparagine residue, preventing the interaction with transcriptional cofactors. Under low oxygen conditions HIF-α is stabilised and translocates into the nucleus where it heterodimerizes with ARNT to form the HIF complex. This complex activates more than 200 direct target genes via binding to *cis*-regulatory regions comprising the consensus 5'-RCGTG-3' sequence, also referred to as hypoxia response element (HRE), which leads to cellular adaptation to hypoxia.

The phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1), also called the Csk-binding protein (Cbp), is an ubiquitously expressed transmembrane adaptor protein. In a microarray study comparing normoxic and hypoxic HeLa cells, we found this gene to be up-regulated. The aim of this thesis was to describe the molecular mechanism behind the hypoxic regulation. We confirmed the microarray data in a panel of additional cancer cell lines from different origin. Furthermore, Pag1 was induced in tissues derived from mice exposed to hypoxia. Genome-wide HIF ChIP-sequencing analysis revealed an HRE 82 kb upstream of the transcriptional start site of the *PAG1* gene. Analysis of the region surrounding this site revealed chromatin modifications like H3K3me1, H3K27ac, overlapping with a DNase I hypersensitivity cluster and binding of a variety of transcription factors. All the aforementioned modifications indicate a potential regulatory region in surrounding the HRE. We confirmed the HIF binding to the HRE by ChIP-qPCR analysis and we detected the binding of the transcriptional coactivator p300. Using reporter gene assays, we independently confirmed that this region consists of a 2 kb enhancer element, responding to HIF binding in hypoxia and specifically regulating *PAG1* but not the neighbouring *FABP5* gene. Mutation of the HRE sequence by site-directed mutagenesis led to the loss of hypoxic induction. We mutated the HRE *in cellula* using the genome editing tool transcription activator-like effector endonucleases (TALEN) in HeLa and MCF-7 cells. No induction of the *PAG1* gene could be observed any longer after mutation of the HRE. By applying chromatin conformation capture (3C) technology we could provide evidence that this enhancer element forms a HIF independent chromatin loop with the promoter region in a cell line specific manner.

To further investigate the impact of HIF-1 and HIF-2 on the transcriptional regulation of *PAG1*, we created HIF-α knockdowns in different cell lines and tested the response of our reporters in these cells. In addition, we overexpressed hydroxylation-insensitive versions of the HIF-α isoforms in the

same cell lines. Our analysis revealed that *PAG1* is HIF-2α specific target gene in the low invasive breast cancer cell line MCF-7 and the pVHL deficient clear cell renal cell carcinoma cell line 768-O. For the other two investigated cell lines, the cervical cancer cell line HeLa and the high invasive breast cancer line MDA-MB-231 *PAG1* seems to be regulated by both HIF-α isoforms. With an overexpression screen of transcription factors, binding to the *PAG1* enhancer region, we wanted to identify HIF interaction partners contributing to the transcriptional regulation of *PAG1*. This further characterisation of the *PAG1* enhancer element did not bring any conclusive results.

PAG1 is an ubiquitously expressed transmembrane adaptor protein, participating in the negative regulation of Src family kinases. It has been shown, that PAG1 itself plays a role in cell signalling processes, independent of Src family kinases. The second aim of this thesis hence was the characterization of the connection between PAG1-regulated Src signalling and cellular adaptation to hypoxia. Our studies using PAG1 knockdown cells revealed a PAG1-independent regulation of Src signalling in hypoxia.

In conclusion, this work provided a functional characterisation of the distal enhancer element regulating the response of the novel hypoxia target gene *PAG1*.

Zusammenfassung

Ein Ungleichgewicht zwischen Sauerstoffversorgung und -verbrauch führt zu einer reduzierten Oxygenierung, auch Hypoxie genannt. Sauerstoffmangel ist ein zentrales Ereignis in einer Reihe von gewissen Krankheiten, wie zum Beispiel Krebs, Arteriosklerose, Hirnschlag oder bei Entzündungsreaktionen. Die Anpassung an Sauerstoffmangel in der Zelle ist durch die Hypoxie-induzierten Transkriptionsfaktoren HIFs gesteuert. Diese Faktoren bestehen aus einem Heterodimer zwischen der permanent exprimierten Form HIF-1β und einer streng sauerstoffregulierten Untereinheit HIF-α. Für den Menschen sind drei Formen beschrieben, HIF-1α, HIF-2α und HIF-3α. Unter normalen Sauerstoffbedingungen hydroxylieren die HIF Prolyl-4-Hydroxylasen (PHD) 1-3 die HIF-α Untereinheit an zwei Prolin Aminosäureresten in ihrer Sauerstoff-abhängigen Degradationsdomäne. Diese Modifikation bewirkt die Interaktion zwischen HIF-α und dem von Hippel-Lindau-Protein, gefolgt vom Proteasomalen Abbau. Zusätzlich zu den PHDs hydroxyliert auch der HIF inhibierende Faktor (FIH) die HIF-α Untereinheit an einem Asparagin Aminosäurerest, welche eine Interaktion mit transkriptionellen Koaktivatoren verhindert. Bei Sauerstoffmangel kommt es zur Stabilisierung der HIF-α Untereinheiten und zu deren Translokation in den Nukleus. Dort bilden sie ein Heterodimer mit HIF-1β. Dieser Transkriptionsfaktorenkomplex bindet an ein *cis*-Element mit der hochkonservierten consensus Sequenz 5‘-RCGTG-3‘, auch bekannt als Hypoxie-responsives Element (HRE). Dieser Vorgang erhöht die Transkription von mehr als 200 direkten HIF Zielgenen.

Das Phosphoprotein, welches mit Glycosphingolipiden angereicheten Mikrodomänen assoziiert ist (PAG1), auch als Csk Bindeprotein bekannt (Cbp), ist überall exprimiert und hat die Funktion eines Transmembranadaptors. In einer früheren cDNA-Microarray Studie, die normoxische mit hypoxischen HeLa Zellen verglich, fanden wir eine erhöhte Expression des *PAG1* Gens in Sauerstoff supprimierten Zellen. Das Ziel der vorliegenden Dissertation war es den molekularen Mechanismus hinter der hypoxischen Induktion des PAG1 Gens zu beschreiben. Wir bestätigten die Ergebnisse des Microarrays mit der hypoxischen Induktion von PAG1 in verschiedenen Krebszelllinien. Ausserdem zeigten wir, dass dieses Gen in Gewebeproben von Sauerstoff-supprimierten Mäusen ebenfalls induziert ist. In HIF-ChIP-Sequenzierungsanalysen, die das gesamte Humangenom abdeckte, wurde eine HIF Bindungsstelle gefunden, die 82 kb von der transkriptionellen Start Stelle (TSS) von *PAG1* entfernt ist. Bei der Analyse dieser Region auf Chromatin-Modifikationen wie H3K3me1und H3K27ac, wurde auch eine Überlagerung mit einem DNase I Hypersensitivitätscluster gefunden. Ausserdem hat sich herausgestellt, dass viele verschiedene Transkriptionsfaktoren in der Region der HIF Bindungsstelle binden. Diese Ergebnisse deuten auf die Existenz eines regulatorischen Elementes in dieser Gegend hin. Zusätzlich zu diesen Ergebnissen haben wir mit ChIP-qPCR Analysen das Binden von HIF in dieser Region nachgewiesen. Dabei haben wir auch Interaktion mit dem Transkriptionsfaktor p300 gefunden. Mit Hilfe eines heterologen Reportergen Systems haben wir herausgefunden, dass in dieser Region ein 2 kb grosser Enhancer liegt, der durch Hypoxie reguliert wird und nur die Genexpression von *PAG1* aber nicht vom gegenüberliegenden Gen *FABP5* steuert. Die Zerstörung der Basensequenz der HRE führte zum Verlust der Hypoxie-abhängigen Regulation. Mit Hilfe von TALEN, einer Genom-Bearbeitungstechnik, haben wir die HRE in zwei Zelllinien, HeLa und MCF-7 mutiert. Nach dieser Prozedur konnten wir keine hypoxische Regulation von PAG1 mehr

feststellen. Ausserdem haben wir uns der 3C Technik bedient und konnten die Ausbildung einer HIF-unabhängigen regulatorischen Chromatininteraktion zwischen dem Enhancer und dem *PAG1* Promotor feststellen.

Wir haben auch untersucht, welchen Einfluss HIF-1 und HIF-2 Faktoren auf die hypoxische Genregulation von PAG1 haben. Zu diesem Zweck erzeugten wir HIF short hairpin RNS Klone in verschiedenen Krebszellinen. Unsere Analysen ergaben, dass *PAG1* ein HIF-2α Zielgen in der Brustkrebszellline MCF-7 und in der renalen Krebszellline 786-O ist. Für die beiden anderen untersuchten Krebszelllinien HeLa und MDA-MB-231 fanden wir eine Regulation, die von beiden HIF-α Formen abhängig ist. Mit Hilfe einer Überexpressionsanalyse von Transkriptionsfaktoren, die im *PAG1* Enhancer binden, versuchten wir potentielle HIF Interaktionspartner zu finden, die an der Regulation von PAG1 beteiligt sein könnten. Jedoch waren die Ergebnisse dieses Screens nicht schlüssig.

PAG1 ist bei der Regulation der Src Kinase Familie beteiligt. Ausserdem konnte gezeigt werden, dass das Protein eine Src unabhängige Rolle in zellulären Signalprozessen spielt. Das zweite Ziel dieser Dissertation war es eine potentielle Rolle für PAG1 in Src abhängigen Signalprozessen und der Adaptation der Zelle an Hypoxie zu finden, die vielleicht durch PAG1 miteinander verbunden sein könnten. Wir bedienten uns PAG1 short hairpin RNS Zellklonen, um eine potenzielle Rolle für PAG1 zu finden. Jedoch konnten wir nur eine PAG1 unabhängige Src Regulation in Hypoxie feststellen.

Diese Dissertation fasst eine funktionelle Charakterisierung des Enhancer Elements zusammen, welches die Induktion des neuen hypoxischen Zielgens *PAG1* steuert.

1. Introduction

1.1. Oxygen in organisms

Oxygen is vital for living cells, as it plays a fundamental role in their metabolism as the final electron acceptor in the oxidative phosphorylation to generate ATP (1). In the current atmosphere oxygen displays 21% of the gas composition (2).

During evolution, different strategies were developed to maximise the absorption of oxygen from the air. In mammals oxygen is absorbed by the lungs. In the alveolae it is transported by diffusion to the blood, where most of it (>98%) binds to haemoglobin in the erythrocytes and a small part (less than 2%) is dissolved in the blood itself. In the periphery, oxygen is released to the tissue. The macro-vasculature of organs provides a rapid blood circulation and the optimal supply of oxygen. Measurements of tissue oxygenation show that the oxygen partial pressure (pO_2) is not the same in every organ. Several units are defined to display the pO_2 (3). The most common one, also used in medicine, is the millimetre of mercury (mmHg) as shown in Table 1.

	mmHg
Air	160
Inspired air (in the tracheus)	150
Air in the alveoli	110
Arterial blood	100
Venous blood	40
Cell	9.9–19
Mitochondria	<9.9
Brain	33.8 ± 2.6
Lung	42.8
Skin (sub-papillary plexus)	35.2 ± 8
Skin (dermal papillae)	24 ± 6.4
Skin (superficial region)	8 ± 3.2
Intestinal tissue	57.6 ± 2.3
Liver	40.6 ± 5.4
Kidney	72 ± 20
Muscle	29.2 ± 1.8
Bone marrow	48.9 ± 4.5

Table 1: normal values of pO_2 in various human tissues, displayed in mmHg in the microenvironment (4).

Hypoxia in the context of various oxygen partial pressures should be seen as reduction of pO_2 below the normal values in human tissues. Organs like the kidney medulla in humans, display naturally a very low oxygen partial pressure with 10 to 20 mmHg, which is important for their physiological function (4,5).

1.2. The impact of hypoxia in high altitude adaptation and in diseases

1.2.1 High altitude adaptation

Medical problems occurring at high altitude (hypobaric hypoxia) are caused preliminary by the low inspired pO_2. The fractional concentration of oxygen is independent of altitude over the range of medical interest. This is the reason, why the reduced inspired pO_2 is determined only by the fall in barometric pressure as shown in Fig.1. The relation between barometric pressure and altitude depends on latitude and the season of the year. Near the equator, the barometric pressure at a given

altitude is higher than in locations more North or South, because the sun is mainly warming the air near the equator. This air creates a larger density, which leads to an increase in barometric pressure. Due to this difference in the pressure-altitude relationship, caused by latitude and seasoning, the "standard atmosphere" was introduced and is also extensively used by aircraft industry (6).

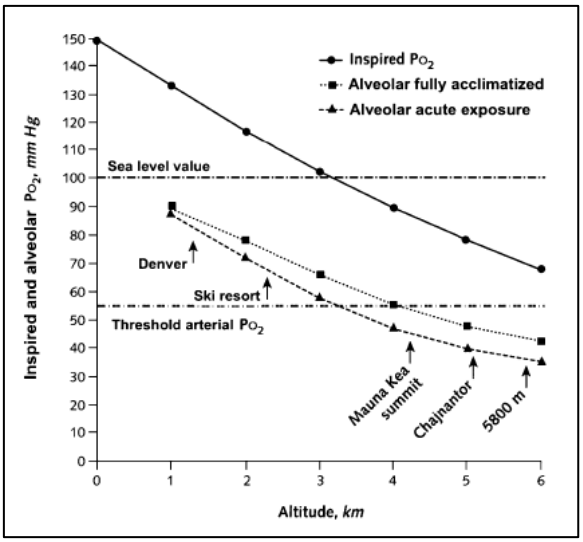

Fig.1: Inspired and alveolar pO_2 values at altitude up to 6000 m (7).

The classic responses to high altitude cover hyperventilation, polycythemia, hypoxic pulmonary vasoconstriction, changes in oxygen affinity of haemoglobin, increase in oxidative enzymes and increase in concentration of capillaries in peripheral muscles (7,8).

The acute response to hypobaric hypoxia encompasses hyperventilation which increases oxygen uptake, cardiac output to deliver more O_2 to the cells and an increase in red blood cell count. The last event is driven by the humoral factor erythropoetin (EPO), stimulated by HIF-2α (hypoxia-inducible factor 2α). Siri and colleagues firstly reported the course of EPO response in humans, measuring the factor in the plasma. After the initial peak, the levels fall to approximately twice sea level values (9).

If acclimatisation and adaptation to high altitude is failing, low landers can develop high altitude mountain sickness (AMS) (10), high altitude pulmonary edema (HAPE) (11), high altitude cerebral edema (HACE) or chronic mountain sickness (CMS). HAPE and HACE can be potentially lethal (12,13).

But also highlanders like Andeans and Tibetans can develop high altitude diseases. These people can develop chronic mountain sickness, caused by severe polycythemia, leading to decreased fluidity of the blood. Tibetans have a lower incidence of CMS than Andeans, consistent with the low haematocrit levels, found in this population. Studies on SNPs (single nucleotide polymorphisms) in the *endothelial PAS domain-1* (*EPAS-1)* gene, encoding HIF-2α, show a loss of function mutation for *EPAS-1* spread in the Tibetan population. These changes in the *EPAS-1* gene could reduce erythropoietic response and further support to avoid the development of CMS (14-16).

1.2.2 Hypoxia plays a crucial role in a variety of diseases

Besides its essential function in high altitude adaptation, further extensive research revealed that hypoxia has also a crucial impact on variety of diseases. Hypoxia plays a crucial role in atherosclerosis, the main source of stroke and myocardial infarction, leading to the main cause of death in the Western world. The atherosclerotic plaque displays a hypoxic core, which is also highly inflamed. Plaque formation is promoted by triggered angiogenesis as the result of a crosstalk between the HIF and nuclear factor of kappa light polypeptide (NF-κB) pathways. This vicious circle leads to progression of atherosclerosis with a potential deadly outcome (17).

Besides atherosclerosis, cancer became a serious thread for the Western countries. After intensive research on the development, progression and treatment of cancer, researchers face a complex and diverse disease with some common molecular features.

In 1930s, Otto Warburg observed altered metabolism in cancer cells. It seemed that cancer cells preferred to aerobic glycolysis than to oxidative phosphorylation, although they did not consume more oxygen than normal tissue, even under normal oxygen circumstances. This observation led to the assumption, that cancer cells have mitochondrial defects (18). However, newer reports show that cancer cells did not sacrifice their oxidative phosphorylation to the enhanced production of lactate (19). Several hypotheses were raised for cancer cells to adapt to fermentative glucose metabolism. First, due to the uncontrollable growth, like in proliferating cells, the metabolism of cancer cells has to adapt to facilitate the uptake and incorporation of nutrients into biomass (20). Second, cancer cells adopting glycolysis gain growth advantages as compared to normal cells. Glycolysis provides an acidic environment, which is harmful to normal cells but does not affect cancer cells, leading to a cellular defence mechanism for cancer cell growth. Third, it was postulated that glycolysis produces less reactive oxygen species (ROS) and so to have a cell protective effect (21). Lastly, it is believed that glycolysis generates ATP faster than oxidative phosphorylation as long as the glucose supply is sufficient, to maintain cellular function and the high proliferation rate (22). HIF-1α was reported to have a certain influence in the maintenance of the Warburg effect. Transcriptional activation of HIF-1α target genes, synergistically promote the Warburg effect, allowing cancer cells to gain growth advantages (23,24). The molecular mechanism leading to this effect will be discussed in a later chapter.

Douglas Hanahan and Robert A. Weinberg summed the results of over a decade of cancer research up in their famous “hallmarks of cancer” review. There also hypoxia reappeared on the stage on cancer research, in the subchapter sustained angiogenesis (25). In 1955, Thomlinson and colleagues showed that tumor cells grow as cord around blood vessels and areas which are more far away than 180 µm from the vessels, display a necrotic region. This chronic hypoxia was caused by the fact that the tumor outgrows its supplying blood vessels. Another feature was also found in the same study, perfusion-limited hypoxia, caused by shutting down of aberrant blood vessels or reversed blood flow. Reopening of these vessels leads to reoxygenation of the surrounding tumor tissue, causing reoxygenation injury. This injury is defined by destruction of tissue due an increase in free radicals, tissue damage and activation of stress response genes (26,27). Hypoxia initially came into the focus

of research because of its impact in radiotherapy. Radiation therapy requires free oxygen radicals which induce severe biological damage in the treated tumor tissue. In contrast, tumor cells in hypoxic regions were resistant to this treatment and even were able to continue proliferation (28). The direct evidence for hypoxia in human cancer came from Peter Vaupel and colleagues, who were the first to measure the pO_2 in a set of human cancers. They also could show that low tumor oxygenation is associated with increased metastatic potential and poor prognosis and outcome for the patient (29-31). So it became more and more important to develop hypoxia markers and methods to measure tumor hypoxia in patients and tumor tissue. In the development of imaging probes for hypoxia markers nitroimidazoles received a lot of attention. 2-nitroimidazoles or azomycin, was originally discovered as antibiotics against anaerobic bacteria and protozoa (32). The antibacterial property of this substance is based on the fact, that nitroaromatics are selectively reduced by nitroreductase enzymes under hypoxic conditions to form hydroxylamine intermediates which can bind irreversibly to nucleophilic groups in proteins or DNA. This feature enables to differentiate between normoxic and hypoxic tissue (33). Injection of derivates such as pimonidazole or EF5 ((2-nitro-1H-imidazol-1yl)–N-(2,2,3,3,3-pentafluoropropyl) actamine) can be used as exogenous hypoxia markers. These compounds can be injected into the patient before surgical resection of the tumor tissue. Immunohistochemical evaluation of the tissue can then provide information about oxygenation status in relation to tissue histology. However these kinds of non-invasive data just provide only a temporal information about the pO_2 of tumors, since due to aberrant vessel formation inside the tumor the oxygenation of the tissue underlies permanent changes. Pimonidazole is considered as the standard hypoxic marker, since the molecule and the antibody to detect it are commercially available. The binding of this marker increases below a pO_2 of 10 mmHg (34).

To measure directly the tumor hypoxia in patients in clinical application positron emission tomography in combination with an injected tracer can be used. The main strategy behind this method is, that the compounds like ^{18}F-fluoromisonidazole (FMISO-PET) or Cu-ATSM (Copper(II)diacetyl-bis(N4-methylthiosemicarbazone) binds to cells in a reduced state, so with low oxygen content. This application is until now the gold standard to identify hypoxic tumors in patients (35).

1.3. The cellular adaption to hypoxia

The master regulators of cellular hypoxic adaptation are the hypoxia-inducible factors (HIFs), consisting of an oxygen-degradable-α and a constitutively expressed β-subunit (36). Under normoxic conditions these factors are hydroxylated by the prolyl-4-hydroxylases domain (PHD 1-3) (37) and the factor inhibiting HIF (FIH) (Fig.2) (38). This protein modification leads to recognition by the von Hippel-Lindau protein (pVHL), which is a part of the E3 ubiquitination machinery and marks HIF-α for proteasomal degradation (39).

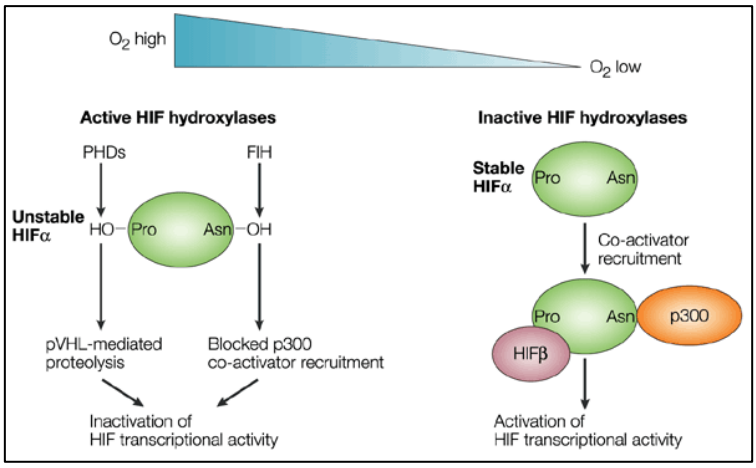

Fig.2: Scheme depicting the oxygen-dependent regulation of HIF-α.

Under hypoxic conditions the hydroxylation enzymes lack one of their substrates oxygen, so no hydroxylation of HIF-α takes place and the α-subunit escapes degradation. HIF-α translocates to the nucleus, heterodimerizes with the HIF β-subunit and recruits additional transcriptional cofactors, like p300 (Fig.2). This transcription factor complex binds to the HIF binding site (HBS) motif 5'-A/G CGTG-3' in the hypoxia response element (HRE) to activate target gene expression (36).

1.3.1. The oxygen-labile HIF-α subunits

There are three different α-subunits known, named HIF-1α, HIF-2α, also called EPAS1 and HIF-3α. All three are composed of a basic helix-loop-helix DNA binding domain (bHLH), two Per-ARNT-Sim (PAS) domains, shared with the *Drosophila* proteins period (Per), the single-minded (Sim) and the human ARNT (aryl hydrocarbon receptor nuclear translocator) protein (Fig.3). bHLH and Pas domains are involved in DNA binding and heterodimerization with HIF-1β, also called ARNT.

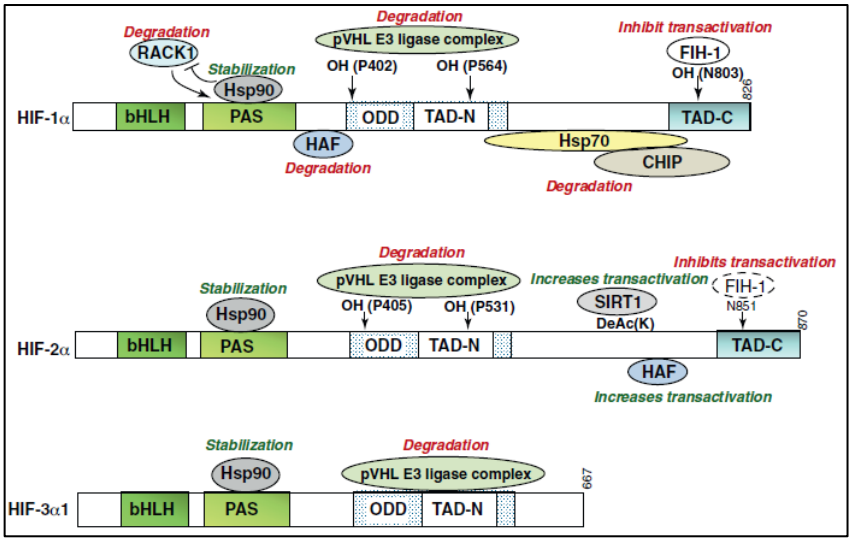

Fig 3: Overview of the oxygen-sensitive HIF-α isoforms, including their known regulatory protein-protein interactors (40).

HIF-1α is the most intensively studied isoform of all three HIF-α subunits, first described and discovered in the regulation of the *erythropoietin gene* (*EPO*). It activates the gene expression via

binding to the 3'enhancer element. This was the first mechanistically described hypoxic gene regulation. The *HIF1A* gene is located on chromosome 14 and is ubiquitously expressed in all tissues. Genetic ablation of this gene in knockout mice shows a non-viable phenotype with embryos dying at E10 containing defects in the cephalic mesenchyme, blood vessels and the presumptive myocardium (41).

The protein is regulated under normal pO_2 by PHD-mediated hydroxylation at two conserved prolyl residues. P402 is located in the oxygen-dependent degradation (ODD) domain, whereas P564 is located in the integrated N-terminal transactivation domain (N-TAD), a part of the ODD domain. These hydroxylated residues form the recognition site for the pVHL. The C-terminal transactivation (C-TAD) domain bears a conserved asparagine residue (N803), the hydroxylation site for FIH. C-TAD and N-TAD are required for transcriptional activation. HIF-1α is most active during short hypoxia (2-24 hours) or intense hypoxia and is important for cellular adaptation. It activates a broad set of target genes, involved in early metabolic adaptation. Under normoxic conditions cells convert glucose to pyruvate, entering the tricarboxylic acid cycle (TCA). This process generates electrons for ATP production via oxidative phosphorylation, where at the end of the electron transport chain oxygen serves as the final electron acceptor. Under hypoxic conditions, this acceptor is missing and cells increase production of lactate from glucose to generate ATP. Most of the genes regulating this switch are HIF-1α targets, *pyruvate dehydrogenase kinase* (*PDK*) and *lactate dehydrogenase A* (*LDH-A*). Besides glycolytic enzymes, glucose transporters and induction of mitochondrial autophagy, also the regulation of the pH is essential. Since lactate production is decreasing the intracellular pH (acidosis), the up-regulation of carbonic anhydrase enzymes is one of the best studied HIF-1α dependent changes (42). The mentioned changes in cell metabolism can also be found in cancer cells promoting the aforementioned Warburg effect. Mutations of the mitochondrial genes *succinate dehydrogenase* (*SDH*) and *fumarate dehydrogenase* (*FH*) lead to an accumulation of their reactant. Succinate and fumarate can inhibit PhDs supporting HIF accumulation (43,44). Together with other mutations, like c-MYC overexpression, HIF supports the Warburg effect in cancer cells (45).

The HIF-2α isoform is encoded by the *EPAS1* gene on chromosome 2 in humans. In contrast to HIF-1α, its expression is restricted to certain tissues, like lung, heart and liver of adult mice and during embryonal development (46). It is active under mild hypoxia or longer duration of hypoxic exposure (24-72 hours). Genetic ablation is also lethal, but compared to HIF-1α, the mouse knockout embryos die at E9.5 to 12.5 and due to defects of larger blood vessels (47). Another phenotype was reported, where the deficiency of this transcription factors leads to bradycardia, marked reduction in norepinephrine production and mid-gestational fetal death (E16.5). *EPAS1* is highly expressed in the chromaffin cells of the organ of Zuckerkandl, the major source of fetal catecholamines. The lethal phenotype could be rescued by giving the mothers L-3,4-dihydroxyphenylalanin (L-DOPA) and D,L-threo-3,4-dihydroxyphenylserine (DOPS). The rescued *EPAS1* pups were born, but died within 24 hours after birth (48). The protein itself has the same protein domain composition as HIF-1α. In terms of regulation HIF-2α is hydroxylated by the PHDs at proline residues P405 and P531, also laying in the ODD domain. FIH hydroxylates the protein at residue N851. The most prominent and most studied HIF-2α target gene is *EPO*. Mutations in the *EPAS1* gene are implicated in polycythemia, a

hematologic disorder of the red blood cell mass (49). Besides, HIF-2α has an important role in angiogenesis and cancer progression (50).

HIF-3α, encoded by *HIF3A* gene on chromosome 19 in humans, is the least studied isoform of all HIF-αs. Compared with its other sister isoforms it is lacking the C-TAD (51). In humans 10 different splice variants of HIF-3α are known. The variant 3-1 is also named the inhibitory PAS (IPAS) protein, first characterised in 2001. Expression analysis with *In situ* hybridisation in the study from Makino and coworkers revealed an expression in mouse cornea, retina and cerebellum. IPAS interacts with HIF-1α, resulting in reduced ability of the HIF/ARNT complex to recognize and to bind to HRE. Furthermore they could reveal an anti-angiogenic effect of IPAS/HIF interaction in the cornea of mice (52). Further experiments with the other splice variants of HIF-3α revealed an unclear picture of function. The human HIF-3α4 was found to form a complex with HIF-1α/ARNT and regulates the transcriptional activity of HIF-1 in a dominant negative manner (53). Knockdown experiments in Hep3B cells investigating the role of the other HIF-3 isoforms show that their lack reduced hypoxic gene induction, whereas other negatively regulate hypoxic gene expression (54). A recently published study shows that the isoform HIF-3α-9 is an oxygen-dependent transcriptional activator, which up-regulates a unique target gene set in human cells and in zebrafish (55).

1.3.2. ARNT/HIF-β

ARNT/HIF-β belongs, like the HIF-αs, to the basic helix-loop-helix transcription factor family. Another binding partner of ARNT is aryl hydrocarbon receptor (AhR), binding as heterodimer to the xenobiotic response element (XRE) with its 5'-GCGTG-3' sequence. Experiments, using conditional ARNT knockout mice, show a prevention of AhR and HIF-1α based gene regulation, referring to a substantial role in both pathways (56). Besides its role as dimerization partner in hypoxic gene regulation, ARNT also plays an important role in different physiological responses like xenobiotic metabolism, vascular development, immunosuppression, T-cell differentiation, reproduction and cell cycle progression (57). In general, ARNT expression is considered to be ubiquitous, constitutive and abundant (58-60). However, there was early evidence, that also ARNT is a hypoxia-inducible protein. In 1995 Wang and colleagues published that ARNT levels are increased in hypoxia on mRNA and protein level (61). Further investigations on this topic revealed evidence for a cell-line specific inducibility of ARNT. Chilov and colleagues found no ARNT alterations in human HeLa, Hep3B and LN229 cells (62). In contrast, Zhong and colleagues demonstrated that ARNT protein levels in PC-3 cells are increased upon hypoxia or cobalt chloride exposure (63). A recent study investigated hypoxia-dependent ARNT regulation in a number of cell lines, including MCF-7 breast cancer cells, HepG2 and Kelly. This study revealed increased ARNT protein levels, but no mRNA up-regulation, suggesting different regulation mechanisms on both mRNA and protein level in cell-type specific manner (64). However, the mechanism behind still remains controversial and unclear.

1.4. The oxygen-dependent regulation of HIFs

1.4.1. The Prolyl-4-hydroxylases

The identification and characterisation of the first HIF prolylhydroxylase was done in *C. elegans*, where it was named egg-laying abnormal 9 (EGL9) (37). In human three orthologous isoforms are identified. All of these enzymes, including also FIH, belong to the 2-oxoglutarate dependent dioxygenase superfamily. They have an absolute requirement for molecular oxygen as co-substrate for the hydroxylation reaction. This provides a direct link between HIF regulation and oxygen availability. Among all three hydroxylases, genetic studies have been shown, that PHD2 is the most important enzyme to regulate HIF-1α. Systemic PHD2 knockout mice die during embryonic development, due to placental defects. In contrast, PHD1 and 3 knockout mice are viable (65). A fourth member of the family PHD4 is not fully characterised yet. Overexpression of this isoform leads to HIF-α regulation, PHD4 is located as transmembrane protein in the ER (66,67).The hydroxylation of HIF-α increases the affinity for the pVHL (39).

Inhibitors blocking the function of PHDs are important tools to mimic hypoxia. PHDs and FIH contain a Fe^{2+} in their active site, which binds the molecular oxygen during the hydroxylation reaction. This metal ion can be substituted by Co^{2+},Cu^{2+},Zn^{2+} and Mn^{2+}. $CoCl_2$ is one of the classic hypoxia-mimicking substances. The mechanism can be explained by the direct inhibition of the HIF hydroxylases due to the aforementioned substitution (68). Deferrioxamine, Clioquinol, Ciclopirox olamine and 3,4-EDHB are iron-chelators, blocking both PHD and FIH function and are used as hypoxia-mimicking substance. The most commonly used inhibitor is Dimethyloxalylglycine (DMOG), which is chemically closely related to the PHD substrate 2-oxoglutarate. This compound was originally discovered to inhibit collagen prolyl-4-hydroxylases, which activity is crucial for confirmation and secretion of interstitial collagen (69). Further research leads to the synthesis of a novel compound JNJ-420419365 (70).

1.4.2. FIH, the factor inhibiting HIF

FIH is the only enzyme hydroxylating HIFs in the C-TAD at a conserved asparaginyl residue (Asn803 in human HIF-1α and Asn851 in human HIF-2α). This modification does not lead to proteasomal degradation, but inhibits the binding of the transcriptional cofactor p300 CH-1 domain (38). Studies using FIH knockout mice show, that these mice exhibit reduced body weight, elevated metabolic rate, hyperventilation, improved glucose and lipid homeostasis, and are resistant to high fat diet-induced weight gain and hepatic steatosis. So FIH plays also an important role in respiration, lipid metabolism and energy balance (71).

1.4.3. Modulation of HIF transcription by other pathways

Until now, very little is known about the integration of pathways on the transcriptional level. Nuclear-factor-κB (NF-κB) regulates the transcription of the *HIF1A* gene, stimulated by T-helper 1 (Th1) cytokines (72). Th2 cytokines interleukin-4 (IL-4) and IL-10 differentially activate *EPAS1* expression,

but the full mechanism has not been described yet (73). The *HIF1A* locus is also regulated by the SWI/SNF chromatin remodelling protein BAF57 (74). Besides transcription there is also modulation on the mRNA level. STAT3 can also elevate HIF-1α levels in a rat liver cancer model (75).The iron response element binding protein 1 (IREB1) binds exclusively to the canonical iron response element (IRE) in the HIF-2α 5'-UTR, inhibiting HIF-2α translation. This mechanism is exclusively regulating HIF-2α, since this isoform is the primary regulator of cellular iron metabolism and erythropoiesis *in vivo* (76).

1.4.4. Posttranslational modification of HIFs

Besides oxygen dependent degradation the HIFα isoforms are also regulated by a broad range of oxygen-independent modifications, influencing their potential in gene transcription in hypoxia and their stability.

Phosphorylation

It is well known that HIF-1α and HIF-2α are phosphorylation targets with certain isoform specificity. Richards and colleagues reported that HIF-1α is a phosphorylation target of the p42/p44 mitogen-activated protein kinases (MAPKs), promoting HIF transcriptional activity and linking hypoxic and growth factor pathways (77). Huang and coworkers reported that HIF-1α represses MYC-dependent expression of the DNA damage repair protein Nibrin (NBS1), by replacing the SP1 transcription factor from the MYC complex (78). The phosphorylation of the HIF-2α protein at T324 by the protein kinase D1 (PKD1) leads to inhibition of Sp1 interaction (78). Furthermore, it has been demonstrated in PC12 cells, that also HIF-2α activity is modulated by MAPK pathway in calmodulin sensitive manner independent of Ras (79). Other phosphorylation events catalysed by casein kinase 1 (CK1) and ataxia telangiectasia mutated (ATM) have been reported to modulated HIF-1α activity (80,81). However if HIF-2α is also a target of these kinases remains unknown.

Acetylation

The mouse arrest defective-1 (mARD1) protein destabilizes HIF-1α via acetylation of K532, an event which can be reversed by recruitment of HDAC1 to HIF-1α via metastasis-associated protein 1 (MTA1) (82,83). Other researchers neither observed an interaction between mARD1 and HIF-1α nor any effects of hypoxia on ARD1 activity. So these results are still under debate (84).

HIF activity is modulated by sirtuins (SIRT), a family of redox-sensitive, NAD+-dependent deacetylases and/ or ADP-ribosyltransferases. Mammalian cells express a number of sirtuins, up to 7, responsible for complex changes in gene expression, metabolism, cellular redox state and other controversial functions (85). HIF-2α transcriptional activity is enhanced *in vitro* and *in vivo* by forming a complex with SIRT1 and deacetylation of conserved lysine residues in the N-TAD of HIF-2α (86). SIRT1 also deacetylates HIF-1α lysine residue 674, blocking p300 recruitment and resulting in transcriptional repression (87). The opposing effect of SIRT1 to HIF-1α and HIF-2α could push cells towards either HIF-1α or HIF-2α transcriptional programs in response to metabolic cellular state and activity of the cell. Park and colleagues proposed a potential positive feedback loop between HIF-1α,

promoting glycolysis and reducing the NAD+/ NADPH ratio under hypoxia. In these conditions HIF-1α activity would be promoted and HIF-2α would be decreased due to SIRT1 inhibition (87). Since both HIF-1α and HIF-2α are binding to the *SIRT1* gene promoter, inducing expression under hypoxia. In addition AKT activity can induce both HIF and SIRT1 expression by downregulating miR199a-5p expression. So the aforementioned feedback loop hypothesis has to be redefined (88,89). HIF activity is also regulated by other sirtuins. SIRT6 is a HIF-1α repressor, showing SIRT6 depletion increases HIF-1α dependent glucose uptake and glycolytic activity at the expense of mitochondrial respiration. The precise mechanism of this interaction is still not fully understood (90). Another indirect mechanism via SIRT3 targeting HIF-1α is the suppression of mitochondrial reactive oxygen species (ROS), which in turn lead to HIF-1α stabilisation. Due to this reason also SIRT3 deficient cells display HIF-1α dependent increase in glucose transport, glycolysis and proliferation (91).

Nitrolysation

The regulation of HIF-αs is not only dependent on oxygen. Other posttranslational modifications have been reported, stabilizing or destabilising HIF-α proteins directly or indirectly. S-nitrosylation connects HIF and NO pathways. Nitrosylation of Cys520, the only cysteine in the ODD domain, prevents binding of the pVHL complex and so proteasomal degradation. In indirect manner nitrosylation of VHL and inhibition of PHDs via NO, competing as substrate with oxygen, leads to normoxic HIF-αs stabilisation (8,92).

SUMOlyation

SUMOylation refers to the conjugation of small-ubiquitin related modifiers (SUMO) to modify protein function and stability. Although only a small fraction of cellular proteins are modified in this manner, it also influences the HIF pathway. HIF-1α can be conjugated with up to 7 SUMO molecules. SUMOylation of the residues K391 and K477 within the ODD domain leads to stabilisation of the protein (93). In contrast, the modification of these residues leads to decreased transcriptional activity (94). These contradictory effects could be explained by side effects of overexpression of parts of the SUMO machinery. Further research has to be performed to solve this issue. Also HIF-2α is a target for SUMOylation. The modification occurs at K394 and decreases its destabilisation and degradation through the proteasome. Also ARNT can be SUMOylated within the dimerization PAS domain, which represses ARNT heterodimerization. For this HIF heterodimer it was shown that this modification does not lead to reduced transactivation activity (95).

HIF-1α regulation via an antisense RNA

A natural antisense transcript of HIF-1α (asHIF) was found to be strictly complementary to the 3'UTR of HIF-1α mRNA, forming duplexes and leading to mRNA degradation (96). It was first discovered in human renal cancer, lymphocytes and human lung adenocarcinoma epithelial cell line (97). Interestingly, there is no antisense RNA for HIF-2α described until now.

Regulation by miRNAs

Besides protein modification or protein-protein interaction another new branch of molecule interaction emerged since the complete sequencing of the human genome, proving the existence of huge classes of non-coding RNAs, also contributing to protein stability and gene expression (98). miRNAs are also regulated by hypoxia, contributing to either a positive or a negative HIF feedback loop modulation (Fig.4) (99).

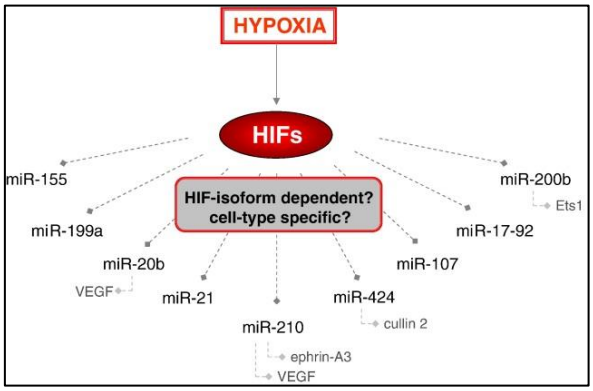

Fig. 4: Examples of miRNAs influenced by HIFs. The activation of some miRNAs (e.g. miR-210) by hypoxia is widespread whereas others (e.g. miR-424) may be regulated in cell-type specific manner (100).

miR-210 is considered as the prototypical hypoxia-induced miRNA (101). It is part of a positive feedback loop in hypoxia, down-regulating PHD activity via decreasing *glycerol-3-phosphate dehydrogenase1-like* (*GPD1L*) levels, by binding in the 3'UTR and therefore decreasing its translation. In hypoxia, miR-210 indirectly increases HIF-1 protein stability via this positive feedback loop (102). Bruning and coworkers showed in Caco-2 cells that HIF-1α expression is directly repressed by HIF-1α induced miR-155, which binds to the 3'UTR of HIF-1α during prolonged hypoxia (103). In H22 liver cells miR-20b participates in a negative feedback loop. HIF-1α decreases miR-20b levels; whereas miR-20b directly targets HIF-1α mRNA, regulating its translation. This feedback loop is expanded in tumor cells, suggesting another level of *vascular endothelial growth factor* (*VEGF*) regulation (104). miR-107, miR-17-92 and miR-519c expression is induced by p53, c-myc and the hepatocyte growth factor (HGF) are hypoxia dependent, but also negatively regulate HIF (105-107). Many of these miRNAs are either HIF-1 or HIF-2α dependent or both, dependent on the cell type.

Phosphorylation		
HIF-1α	MAPK	↑
HIF-2α		↑
HIF-1α	(PAS-B T322) PKD1 (PAS-B T324)	↑
HIF-2α		↓
HIF-1α	CK1 (Ser247)	↓
HIF-1α	ATM (Ser696)	↑
Acetylation		
HIF-1α	mSRD1 (K532)	↓
HIF-1α	SIRT1 (K674)	↓
HIF-2α	SIRT1	↑
Nitrosylation		
HIF-α	(Cys 520)	↑
SUMOylation		
HIF-1α	(K391, K477)	**n.d.**
HIF-2α	K394	↑
Anti-sense RNA		
HIF-1α	3'UTR	↓
miRNAs		
HIF-1α	miR-210,	↑
HIF-1α	miR-155, miR-20b, miR-107, miR17-92, miR-519	↓

Table 2: Summary of posttranslational modifications of HIF-1α, HIF-2α or both (HIF-α).
Modifier and protein modification with residue in brackets are indicated if reported. Effect on transcriptional activitiy of HIF are indicated with arrows (↑: increase in activity, ↓: decrease in activity**, n.d.**: not determined).

1.5. The HIF transcriptome

1.5.1. The hypoxia response element (HRE)

The HIF heterodimer binds to a defined sequence in the DNA of the target genes. The core of this sequence consists of the HIF binding site (HBS) with a well conserved 5'-RCGTG-3' motif.

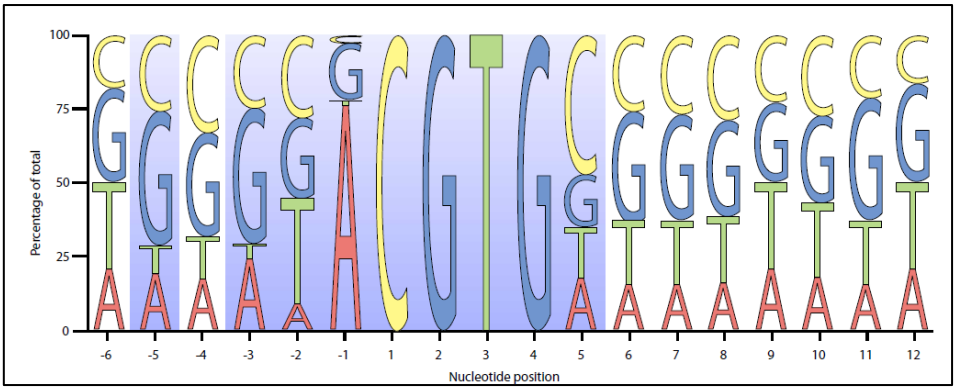

Fig 5: Relative occurrence analysis of the nucleotide position of the core HRE (RCGTG, where R can be A or G) from 108 core HREs. Nucleotide presence is displayed as percentage of total. Shadowed boxes in light blue indicate the non-randomly distributed nucleotides (108).

Frequency analysis shows a strong presence of A>G>C>T at the -1 position of the HRE. This core consensus motif containing 5 nucleotides occurs over 1 million times across the human genome (109). These data indicate that the HBS is necessary, but not sufficient for hypoxic gene regulation. The neighbouring sequence normally consists of binding sites for transcriptional co-activators, which build the full functional hypoxia response element (HRE). The aforementioned occurrence leads to the problem that basically in every gene locus a HRE should be present. But sequence analysis and reporter assays do not distinguish a direct or an indirect target genes, they just analyse sequences. To fully characterize the HIF transcriptome a different way of analysis was necessary. Novel approaches

using chromatin immunoprecipitation (ChIP)-sequencing and ChIP-chip techniques were applied to analyse HIF binding over the whole genome and were combined with transcriptome analysis (110-113). Schödel and colleagues study from 2011 is the only genome-wide study comparing HIF-1α and HIF-2α binding sites distribution, showing that HIF-1α binds more promoter proximal than HIF-2α, which binds preferentially distal from promoter regions. 60% of all HIF-1α binding sites are located outside of the 2.5 kb around known transcriptional start sites (TSS). In comparison to HIF-2α, in which case the number is up to 80%. For other transcription factors, like p53 and estrogen receptor, the binding to promoter distal elements like enhancers are also reported. This could potentially contribute to cell-type specific gene regulation (114,115). The comparison of HIF binding sites in the low invasive MCF-7 breast cancer cell line and the clear cell renal cell carcinoma (ccRcc) cell line 786-O revealed that promoter proximal sites are more likely to be shared between cell lines, compared to distal sites (110).

Genome-wide ChIP experiments identified the HRE consensus sequence in approximately 75% of all HIF-binding regions. The absence of the binding motif in 25% of HIF binding sites raised the first hypothesis about the DNA recognition site of the HIF heterodimer, including that there could be another HIF-DNA binding motif existing or that HIF binds also other sequences with low stringency (116). The second hypothesis is that several enhancer regions, each binding one transcription factor, but all interacting with the same promoter and so indirectly interacting with each other. Additionally, the ENCODE consortium showed that each promoter interacts with ca. 3.9 enhancer sites across the genome (117).

Besides, also the surrounding of HIF binding site is essential. Analysis of the chromatin structure revealed that the majority of HIF binding sites as defined in hypoxia, correlates with sites of open chromatin in normoxia, as shown by DNaseI hypersensitivity cluster or nucleosome-sequencing (111,113). For example, the *CCND1*, encoding Cyclin D1 related enhancer in the VHL defective renal cell line 786-O has a constitutively accessible confirmation. Reintroduction of VHL leads to closure of the chromatin structure (110). These findings led to the assumption that the locus covering the HRE is already open in normoxia and not just opened due to the hypoxic stimulus.

Studies by Xia and Kung (2009) as well as Tanimoto and colleagues (2010) showed that HIF binding sites are associated with high levels of histone H3K4me3, a mark for active promoters. No overlap was observed with H3K27me3, a mark for inactive chromatin (109,113). HIF-DNA binding can also be impaired by methylation of the CpG nucleotide in the RCGTG motif. So, also the methylation status of the DNA has to be taken into account (118). Close to HIF consensus motif also other recognition sequences of transcription factors are enriched. These motives include the binding motifs of AP-1, cAMP responsive element-binding protein (CREB) and the CCAAT-enhancer binding protein motif (CEBPB) (111,119).

The recruitment of RNApol2 to the promoters of hypoxically regulated genes is increased in hypoxia. A recent study shows that HIF itself is involved in the release of promoter-paused RNApol2 at the promoter of these genes. ChIP-qPCR analysis showed a clear difference in RNApol2 distribution and travelling ratios on gene bodys of direct HIF target genes (120).

1.5.2. Co-operation with other transcription factors for transcriptional activation

HIF-α is the essential factor required for HIF target gene activation. This was widely shown by HIF-α and ARNT/HIF-β knockdowns, blocking or reducing target gene activation. However, other transcription factors are also involved in hypoxic target gene activation. These factors act via direct protein-protein interaction with HIF-α in the transcriptional complex, demonstrating that these factors are part of the HIF enhanceosome. These findings reveal that on the level of target gene activation a crosstalk between various other pathways and hypoxia takes places, which could have important impact on physiological and pathophysiological processes (121). In a lot of reported cases the recruited transcription factors show a distinguishable interaction with either HIF-1α or HIF-2α, showing that distinct enhanceosome complexes exist.

1.5.2.1. Transcription factors interacting with HIF-1α and HIF-2α

The transcriptional co-activator p300/CBP plays an essential role in the activation of hypoxic downstream targets. It binds to C-terminal transactivation domain of HIF-α (122). The binding is inhibited by the asparagine residue hydroxylation N 803 of HIF-α by FIH (38).

The E-twenty-six (ETS) family member ETS translocation variant 4 (ETV4) has recently been discovered as a HIF interaction partner. ETV4 interacts with HIF-1α and p300 as an activation complex for the PHD2 and transferrin genes regulation in hypoxia. In fluorescence resonance energy transfer (FRET) analysis ETV4 also interacts with HIF-2α (123).

1.5.2.2. HIF-1α interacting transcription factors

The Sp1 factor is a zinc finger transcription factor involved in basal transcription of housekeeping genes (124). Sp1 interacts with HIF-1α and facilitates the promoter activation of a broad range of hypoxically regulated genes like *EPO, VEGF, cyclooxygenase 2* (*COX-2)* and *UDP-glucose dehydrogenase* (*UGDH*) (125-127).

ATF-1, a member of the CREB family, binds together with HIF-1α to the promoter of *uncoupling protein 3* (*UCP3*) in muscle cells. (128).

The activator protein (AP-1) transcription factor family is involved in regulation of a large number of genes. AP-1 is composed of dimers formed by Jun (c-Jun, JunB and JunD), FBJ murine osteosarcoma viral oncogene homolog (Fos) (c-Fos, FosB, Fra1 and Fra2), activating transcription factor (ATF) (ATF2, ATF3/LRF1, B-ATF, JDP1 and JDP2) and musculoaponeurotic fibrosarcoma (MAF) (c-Maf, MafB, MafA, MafG/F/K and Nrl) (129,130). Hypoxia has been shown to activate AP-1 and mediate gene expression. Reported genes, which are at least partially induced by AP-1 in hypoxia, include *tyrosine hydroxylase* (*TH*) and *endothelial NOS* (*eNOS*) (131-133). Further studies were performed on the interaction between c-Jun and HIF-1α showing transcriptional activation of HIF

target genes like *VEGF* (134). Moreover other factors of the AP-1 family, like JunB and members of the Fos family, show to participate in hypoxic gene transcription (123,135).

Interferon regulatory factor-1 (IRF-1) activates the transcription of interferon-α and –β target genes. Tendler and co-workers demonstrated that hypoxia increases the activity of IRF-1.They also show interaction between HIF-1α and IRF-1. This complex bind to the regulatory region *of nitric oxide synthetase 2* (*NOS2*), leading to enhanced hypoxic activation of *NOS2* in IRF-1 primed macrophages in hypoxia (136).

The members of the transforming growth factors-β (TGF-β) signalling pathway are often implicated in cancer and cellular de-differentiation processes. TGF-β is also reported to functionally interact with HIF-1 and HIF-1α target genes via Smad protein, which mediate TGF-β dependent gene activation via binding to a subset of transcription factors, like forkhead activin signal transducer (FAST) and ATF (137-139). HIF-1α and Smad physically interact and cooperatively activate transcription of HIF target genes like *VEGF* and the *endoglin gene* (*ENG*) (140,141).

Wnt signalling is mediated by β-catenin that binds to T cell-specific transcription factor (TCF) and activates TCF genes. Chronic hypoxia is known to up-regulate β-catenin in human macrophages via activation of AKT and deactivation of glycogen synthase kinase-3β (GSK-3β), the negative regulator of β-catenin (142-144). SRC dependent phosphorylated form of β-catenin (pY654) interacts with HIF-1α and activates target genes involved in EMT transition like *Snail* and *Twist* (145,146). Hypoxia itself can increase SRC kinase activity through ROS generation, which leads to increase in β-catenin pY654 phosphorylation and up-regulation of target genes (147).

The signal transducer and activator of transcription-3 (STAT3) is activated in hypoxia (148). In the renal cell carcinoma cell line RCC4 and in the highly invasive breast cancer cell line MDA-MB-231, the efficient hypoxic response of HIF-1α dependent target genes requires STAT3 (149). Furthermore, Pawlus and colleagues showed that HIF-1α bHLH and PAS domains are required for binding STAT3 (150).

1.5.2.3. Factors uniquely interacting with HIF-2α

V-ets erythroblastosis E 26 oncogene homolog (ETS) has been demonstrated to specifically interact with HIF-2α for a number of target genes. The complex containing ETS-1 is necessary for sufficient hypoxic induction of *KDR* (VEGF receptor-2). This interaction is mediated by the HIF-2α N-TAD and the ETS-1 exon VII protein domain. HIF-1α is also able to interact with ETS-1, but this interaction is not functional (151). Another factor belonging to the ETS family, ELK-1, has been reported to be phosphorylated by MAP-kinases upon hypoxia. ELK-1 is required in Hep3B for the activation of HIF-2α specific genes including *CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain* (*CITED-2*)*, EPO* and *serpin peptidase inhibitor clade E 1* (*SERPINE 1*), and for the HIF-2 preferential targets like *insulin-like growth factor binding protein 1* (*IGFBP-1*)*, adrenomedullin* (*ADM*) and *N-MYC downstream regulated 1* (*NDRG-1*) (152). Further studies in MCF-7 cells show that a specific HIF-2 dependent subset of genes needs ELK-1 for activation. Promoter analyses of these

genes revealed at least one HRE in close proximity to an ETS-1 binding site in 10 out of 11 genes (153).

The upstream stimulatory factor 2 (USF-2) is a ubiquitously expressed transcription factor. Pawlus and co-workers demonstrated that USF-2 is a global compound of the HIF-2 enhanceosome including CBP and RNA polymerase II. USF-2 is required for the recruitment of the histone acetyltransferase CBP and p300. HIF-2α interacts with its N-TAD domain with USF-2. Inhibiting USF2 expression or USF-2/HIF-2 interaction leads to the expected reduction in tumor growth and associated metastasis (150).

HIF-2α was shown to interact with NF-κB essential modulator (NEMO), which was first found in a yeast-two-hybrid Screening and then verified by co-immunoprecipitation experiments in cells. The study shows that NEMO interacts with the C-TAD of HIF-2α and enhances target gene transactivation by mediating the recruitment of p300 (154).

The hepatocyte nuclear factor 4 (HNF4) is a constitutive factor in liver cells. Together with HIF-2α it is essential for efficient transcriptional activation of *EPO* in liver cells. HNF4 binds the consensus sequence TGACCT in the 3'*EPO* enhancer region downstream of the HRE. HNF4 physically interacts with ARNT of the HIF heterodimer (155).

Transcription Factor	HIF-1α	HIF-2α
CBP/p300	+	+
ETV4	+	+
Sp1	+	-
ATF-1	+	-
AP-1	+	-
IRF-1	+	-
TGF-β	+	-
β-catenin (pY654)	+	-
STAT3	+	-
ETS-1	-	+
ELK-1	-	+
USF-2	-	+
NEMO	-	+
HNF4	-	+

Table 3: Summary table of co-operation of HIFs with other transcription factors.
Interaction between proteins are indicated with **+**, no interaction is expressed with **-**.

1.5.3. Cellular hypoxic adaptation

During prolonged hypoxia (>24 hours, *in vitro*) a change between the prominent HIF-α isoforms takes place (Fig. 6). HIF-1α protein levels are reduced; while HIF-2α levels are induced and remain high. This so called "the HIF-α switch", which leads to changes in target gene activation and is essential for cellular adaptation to prolonged hypoxia.

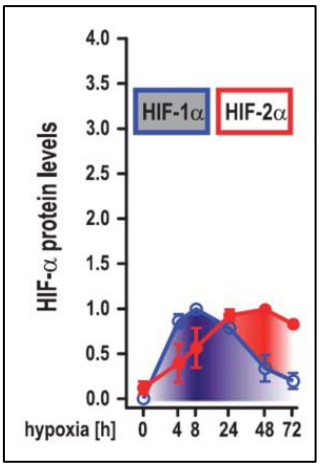

Fig.6: The switch from HIF-1 to HIF-2 dependent transcription during hypoxia duration.
The temporal regulation of HIF-1α is indicated in the blue line, HIF-2 levels in red, from (156).

Besides the aforementioned regulation of HIFs by pVHL, the two following HIF-1α specific ubiquitin ligases participate in the HIF switch regulation.
HIF-associated factor (HAF) binds and destabilizes HIF-1α in normoxic and prolonged hypoxic conditions in an oxygen-independent manner. HAF has no effect on the stability of HIF-2α, but it binds to a distinct C-terminal region, leading to increased transcriptional activity (157).
Additionally, the heat shock protein 70 (HSP70) binds via its carboxy-terminus to HIF-1α. This interaction leads to recruitment of Hsc70-interaction protein (CHIP), a chaperone-dependent E3 ubiquitin ligase, which mediates the ubiquitination of HIF-1α. HIF-2α is not regulated by these proteins under prolonged hypoxia in cultured cells (158).

PHD2 and PHD3 are direct HIF-1α target genes (108). The increase of these PHDs partially compensates for the decreased hydroxylation activity under low oxygen conditions. This negative feedback loop leads to decreased HIF-1α levels in the duration of hypoxia, with the consequence of shortened hypoxic response and duration (159). In the case of re-oxygenation, these elevated PHD2 and 3 levels lead to acceleration of HIF degradation (160,161).

The consequence of a stronger regulation of HIF-1α, in comparison to HIF-2α under prolonged hypoxic conditions is an important change in the major driver of hypoxic response, leading to changes in target gene activation.

1.6. HIF target genes

HIFs integrate a number of different cellular signalling pathways which are physiologically important for cellular adaptation to hypoxia. Till today, more than 200 hypoxia inducible HIF target genes are known, which can be sub-grouped, independently of their HIF specificity to reduce the complexity, in related cellular pathways (Fig.7).

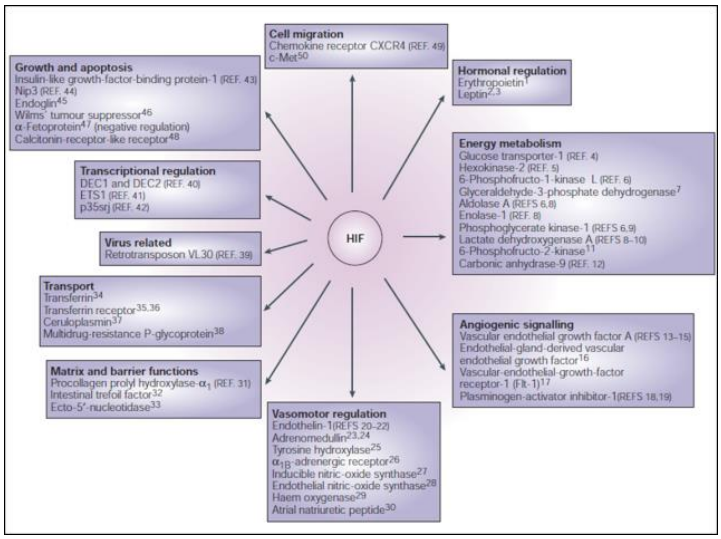

Fig. 7: Activation of HIF integrates a huge subset of pathways.

Overview of proteins, which could be shown to be direct HIF target genes (68).

As indicated in Fig.7 hypoxia dependent gene regulation is implicated in a broad range of physiological and cellular features. HIF-1α and HIF-2α share a lot of commonly regulated genes, dependent on the cell type. But, they also have unique targets as depicted in Table 2. HIF-1α target genes are often implicated in glycolysis and early adaptation to hypoxia. HIF-2α targets are involved in angiogenesis, stem cell maintenance and late adaptation to hypoxia (162). By swapping protein domains between the two HIF-α isoforms, several groups demonstrated that the transcriptional specificity resides in the amino-terminal transactivation domain (N-TAD), suggesting an interaction with isoform specific transcription factors, for gene selectivity (152,156).

Gene (protein)	Function	HIF1α target gene	HIF2α target gene	Cell type
SLC2A1 (GLUT1)	Glucose transport	+	+	RCC[27] and mouse ESCs[36,37]
PLIN2 (ADRP)	Lipid metabolism	+	+	RCC[27]
CA12 (CAXII)	pH homeostasis	+	+	RCC[27]
FLG (filaggrin)	Cytoskeletal structure	+	+	RCC[27]
IL6 (IL-6)	Immune cytokine	+	+	RCC[27]
ADM (adrenomedullin)	Angiogenesis	+	+	RCC[27]
VEGFA (VEGFA)	Angiogenesis	+	+	RCC and Hep3B cells[27–29]
	Angiogenesis	+	–	Mouse ECs[31] and mouse ESCs[36,37]
BNIP3 (BNIP3)	Autophagy and apoptosis	+	–	RCC[28]
HK1 (hexokinase 1)	Glycolysis	+	–	Mouse ESCs[36,37]
HK2 (hexokinase 2)	Glycolysis	+	–	RCC[27] and mouse ESCs[36,37]
PFK (phosphofructokinase)	Glycolysis	+	–	RCC[27] and mouse ESCs[36,37]
ALDOA (ALDA)	Glycolysis	+	–	RCC[27] and mouse ESCs[36,37]
PGK1 (PGK1)	Glycolysis	+	–	RCC[27] and mouse ESCs[36,37]
LDHA (LDHA)	Glycolysis	+	–	RCC[27] and mouse ESCs[36,37]
NOS2 (iNOS)	NO production	+	–	Macrophages[49]
ABL2 (ARG)	Inhibitor of NO production	–	+	Macrophages[49]
EPO (erythropoietin)	Erythropoiesis	–	+	Kidney[41,42,65] and liver[188]
POU5F1 (OCT4)	Stem cell identity	–	+	Mouse ESCs[155]
SCGB3A1 (secretoglobin 3A1)	Growth-inhibitory cytokine?	–	+	NSCLC[26]
TGFA (TGFα)	Growth factor	–	+	RCC[28,189]
CCND1 (cyclin D1)	Cell cycle progression	–	+	RCC[28]
DLL4 (DLL4)	NOTCH signalling and EC branching	–	+	Mouse ECs[53]
ANGPT2 (angiopoietin 2)	Blood vessel remodelling	–	+	Mouse ECs[53]

ADRP, adipose differentiation-related protein; ALDA, fructose-bisphosphate aldolase A; ARG, Abelson-related gene protein; CA, carbonic anhydrase; DLL4, delta-like 4; EC, endothelial cell; ESC, embryonic stem cell; GLUT1, glucose transporter 1; IL, interleukin; iNOS, inducible nitric oxide synthase; LDHA, lactate dehydrogenase A; NO, nitric oxide; NOS, nitric oxide synthase; NSCLC, non-small-cell lung cancer; PGK1, phosphoglycerate kinase 1; *PLIN2*, perilipin 2; RCC, renal cell carcinoma; TGF, transforming growth factor; VEGFA, vascular endothelial growth factor A.

Table 4: Representative list of shared and unique target genes regulated by HIF-1α and HIF-2α, from (162).

Table 4 shows only a few examples of the more than 200 direct HIF target genes known. Some genes are uniquely regulated by one HIF-α isoform, like *EPO*, regulated by HIF-2α in the kidney or in the liver. In contrast to the *EPO* gene, *NOS2* is an exclusive HIF-1α target gene in macrophages. For most of the direct target genes, the HIF-α isoform dependent regulation is cell type and tissue specific. A shared target is the glucose transporter Glut1, encoded by the *solute carrier family 2 (facilitated glucose transporter), member 1(SLC2A1)* gene. This ubiquitously expressed glucose transporter is responsible for the basal glucose uptake in most tissues. During hypoxia, Glut1 is up-regulated on the mRNA and protein levels. Increased Glut1 maintains enough glucose supply to rescue cellular ATP production by anaerobic glycolysis (163). Therefore, Glut1 is commonly used as a hypoxic control gene in cell culture. Hypoxia causes a change in intracellular pH due to the metabolic switch from oxidative phosphorylation to glycolysis with lactate production. Another group of hypoxic target genes that is involved in the regulation of pH homeostasis are carbonic anhydrases. These transmembrane proteins catalyse the reversible hydration of carbon dioxide to carbonic acid, which stabilises the intracellular pH. The member *carbonic anhydrase 9 (CAIX)* is a well reported HIF target gene (164). The *SERPINE 1* gene, encoding for the plasminogen activator inhibitor 1 (PAI-1), a physiological inhibitor of tissue-type and urokinase-type plasminogen activators. The first study showing that PAI-1 is a direct HIF target was performed in rat hepatocytes (165). Kietzmann and colleagues demonstrated PAI-1 mRNA up-regulation and an increase in secreted protein in cell culture supernatant. They identified an HRE in the *SERPINE1* promoter region of the rat gene. Further studies using human cancer cells lines confirmed PAI-1 hypoxic regulation (166,167). *CAIX* is commonly used as hypoxia control target gene in human cancer cell lines and human cancer tissues as HIF-1α specific targets. PAI-1 is used as HIF-2α control target in the low invasive breast cancer cell line MCF-7; also *CITED-2* is used as HIF-2α control in different cancer cell lines (156,168).

Different approaches to physically identify HIF-DNA binding by ChIP sequencing or ChIP-chip, lead to the identification of novel HIF target genes (109-113). Based on these studies a hypoxic regulation mechanism for the previously identified HIF target gene *CCND1* was found, showing HIF-2 binding to an enhancer element located 200 kb upstream of the TSS in 786-O cells. HIF binding was impaired by a SNP variant in this enhancer region leading to a protective haplotype in this type of clear cell renal cell carcinoma (110). Another genome-wide HIF binding study identified *SLC2A3*, encoding Glut3, as cooperative HIF and lysine (K)-specific demethylase 3A (KDM3A) target in HUVEC cells (112). Follow up studies identified *sperm associated antigen 4* (*SPAG4*) as new HIF target gene and confirmed again *angiopoitin-like-4* (*ANGPL4*) as HIF regulated gene (169-171).

1.6.1. HIFs regulate non-coding RNAs

A novel emerging field of research focuses on RNA species which are non-translated RNA. This class can be subgrouped into short non-coding RNAs with a length below 200 nucleotides, indicating micro RNAs (miRNAs), small nuclear/nucleolar (snRNAs), piwi-interacting RNAs (piwiRNAs) and transfer RNAs (tRNAs). Another group are long non-coding RNAs (lncRNAs), with a length over 200 nucleotides. Non-coding RNAs have important regulatory functions in the cell. Recent studies

demonstrate altered non-coding RNA expression in many cancer cell types implicated in cell cycle regulation, apoptosis, carcinogenesis and metastasis (172). A genome-wide RNA-sequencing study by Choudhry and colleagues in MCF-7 cells showed that all classes of non-coding RNAs are regulated by hypoxia. snRNAs, tRNAs and piwiRNAs showed an overall downregulation in hypoxia. In contrast, mRNAs, lncRNAs and miRNAs show an up-regulation. Among the lncRNA *nuclear paraspeckle assembly transcript 1 (NEAT)* was shown to be a HIF-2α specific gene and *metastasis associated lung adenocarcinoma transcript 1 (MALAT)* was identified to be HIF regulated (120). However, very little is known about the role of non-coding RNA in hypoxia.

1.7. The interplay between HIFs and oncogenic signalling

Oncogenic and hypoxic pathways show a strong overlap between their activated target genes; furthermore HIF-1 and HIF-2 clearly influence tumor progression. Both are able to induce essential genes involved in angiogenesis, proliferation, glycolysis, immortalisation and genetic instability, pH regulation and the shut-down of growth inhibitory signals. The direct interplay between these pathways takes place on different cellular levels, especially up-stream of the HIF transcription factors, or at the level of protein-protein interaction.

1.7.1. HIFs and MYC

In many cell types hypoxia supresses proliferation. Stabilisation of HIF-1α leads to cell cycle arrest by influencing MYC activity in a direct or indirect manner. MYC is a bHLH/leucine zipper transcription factor, which is overexpressed in more than 30% of human cancers. The function of MYC is to control the G1/S transition in the cell cycle by forming heterodimers with the MAX protein and promoting the expression of cell cycle progression genes (173). HIF-1α directly binds MYC via protein-protein interaction, leading to a release of MYC-interacting Zn protein 1 (MIZ1). MIZ1 increases p21 expression and induces cell cycle arrest (174). The direct binding of HIF to MYC causes the release of the both shared transcriptional cofactor Sp1, blocking MYC target gene expression (175). In this regard, the inhibition of MYC through HIF-1α binding to Sp1 is implicated in genomic instability, which has been suggested to be a direct consequence of MYC overexpression (176). Studies, using different cell models and varying extents of hypoxia conditions did not show the aforementioned effect. So the inhibitory effect of HIF on MYC by releasing Sp1 is still under debate. HIF-1α mediates the induction of Max interactor 1 (MXI1), which interacts with the MAX binding site to inhibit the expression of *ornithine decarboxylase 1* (*ODC1*) and *peroxisome proliferator-activated receptor-γ coactivator 1β* (*PGC-1β*) supressing mitochondrial function. MAX can be directly bound by HIF-1α, so the interaction supresses MYC activity by competing for MAX against the binding of HIF-1α (45). It was reported that HIF-1 decreases MYC activity by attenuating MYC protein levels through a proteasome-dependent pathway, with unknown components (177). Due to these multiple mechanisms and due to the fact that HIF-1 drives the glycolytic switch, it is able to effectively supress MYC dependent effects on anabolic metabolism, protein synthesis and cell cycle progression as an important feature of hypoxic adaptation (177,178). A summary of the transcriptional interaction of HIF-1α and HIF-2α is depicted in Fig. 8.

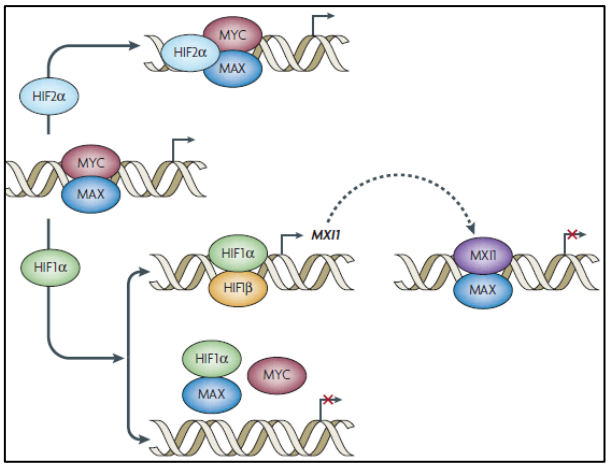

Fig.8: The HIF-1α, HIF-2α and MYC protein-protein interaction transcriptional network.
Interaction with HIF-2α increases MYC activity. HIF-1 repressive function is displayed by transcriptional induction of MXI1 or competition for MAX.

In contrast to HIF-1α, HIF-2α seems to collaborate with MYC (Fig.8). Transformed cells, expressing HIF-2α, show enhanced MYC activity, with increased G1/S-phase transition including increased levels of MYC target genes and elevated MYC promoter occupancy. HIF-2α promotes cell cycle progression in hypoxic cells having transcriptional effects on both MYC activated and repressed genes via interaction with MAX, Sp1, and MIZ1 (179). This interplay has an important role in the neoplastic progression of renal clear cell carcinomas (RCCs), after the loss of a functional pVHL tumor supressor. These cells display a reduced genomic instability, which correlates with the MYC-dependent gene expression of DNA repair proteins, like breast cancer 1 (BRCA1) and the mitotic checkpoint serine/threonine kinase BUB1 (180). Both HIFs are interacting with MYC but with a different outcome. HIF-1α has a suppressive effect, whereas HIF-2 promotes MYC activity.

1.7.2. HIFs and p53

Environmental stresses in tumors lead to activation of p53, the so called “guardian of the genome”, which is mutated in most of the cancers. In normal cells the levels of p53 are kept very low, due to murine double minute 2 (MDM2)-mediated degradation. The functional p53 homotetramer is stabilized due to different stress stimuli, including abnormal proliferation signals, osmotic stress, DNA damage, replication stress and also hypoxia. Upon stabilisation p53 is phosphorylated by ataxia telangiectasia mutated (ATM) protein and activates its target genes inducing to apoptosis. Several studies have shown that p53 accumulation occurs within hypoxic regions of solid tumors, correlating with cells undergoing apoptosis and/ or accompanied by acidosis and nutrient deprivation (181-183).

Studies show, that hypoxia increases p53 protein levels. The accumulation increased with duration of the hypoxia as well as the decrease of pO_2 (184). It was proposed that HIF-1α is required for induction of p53 upon hypoxia. p53 and HIF-1α physically interact with each other, through the ODD and the N-TAD domain in anoxic conditions (185,186). Further studies disproved the model of a HIF-dependent p53 induction and a direct interaction between the two proteins (187). Several HIF-independent pathways have been shown to induce p53 under severe hypoxic conditions. MDM2 is downregulated

in hypoxia, leading to sustained stabilisation of p53 (188). Hammond and colleagues show in their study that p53 is induced in hypoxia due to induction of replication arrest (189). Furthermore also MDM2 independent mechanisms were found to induce p53. Inhibition of the p53 specific phosphatase-1 increases its activity (190). Besides an increase in ROS levels leads to induced p53 levels (191). In addition to MDM2, HSP70 participates in p53 degradation. HSP70 is downregulated upon hypoxia, leading to increased p53 protein levels (192). Increased levels of p53 usually lead to apoptosis. This holds also true of an increase under hypoxia, but also cases with no induction of apoptosis are reported (193). The increased amount of p53 fails to induce its target genes, like *BCL2-associated protein X* (*BAX*) and *BCL2 binding component 3* (*BBC3*). p53 was found to be bound in the promoter region of these genes, but no transactivation occurred (194). No recruitment of CREB-binding protein (CBP) or the cofactor p300 could be observed. HIF-1α and p53 could maybe compete against the transcriptional cofactors. Another explanation for this observation could be that hypoxia changes the posttranslational modification of p53 to one, which recruits co-repressors (195). It should be also taken into consideration that a hypoxic induction of p53 could be only observed in some cell lines not only under hypoxic conditions, but in anoxic conditions. Furthermore it could be shown that hypoxia has no effect or even decreases p53 levels. The conflictive observations can be explained by the facts, that these effects are cell line specific. In addition the use of different conditions in the duration and severity of hypoxia/anoxia contributes to different study outcome (196).

p53 has also an effect on HIF-1α. Studies with p53 deficient cells revealed higher HIF-1 levels compared to wild type cells. An involvement of MDM2 was suggested to participate in this mechanism, but studies revealed that MDM2 is not involved in HIF protein degradation (197). The same observation was made *in vivo*. p53 knockout mice display higher HIF-1α levels in hypoxia compared to control mice. However this effect just can be observed, when severe cellular stress occurs (198). The molecular mechanism behind the influence of p53 on HIF-1α could not be clarified yet.

HIF-1α and p53 regulate some common pathways in a synergistic or contradictory manner. The two transcription factors synergistically cause cell cycle arrest, by induction of p21 expression (199). In angiogenesis the regulation is contradictory. HIF-1α induces growth of new blood vessels by increasing the expression of several target genes, like *VEGF,* nitric oxide synthase *(NOS), fibroblast growth factor (FGF2)* and *matrix metalloproteinases* (*MMPs*). p53 inhibits angiogenesis with three different mechanisms. First, by increasing the production of anti-angiogenic factors, among them *thrombospondin-1 (THBS1)*. Second p53 directly inhibits the HIF-pathway. Finally, p53 can transcriptionally repress angiogenic target gene expression, like *VEGF* and *FGF* (200). A scheme giving an overview over the current influence of p53 and HIF-1α under different oxygen conditions is shown in Fig. 9.

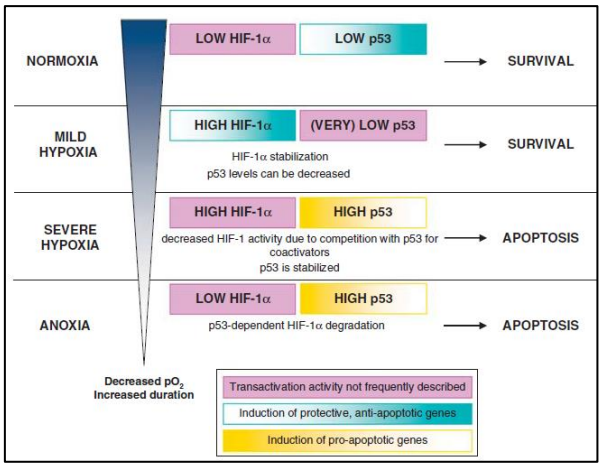

Fig.9: Model for the regulation of HIF-1α on p53 and vice versa, dependent of the severity and the duration of hypoxia, from (195).

HIF-2α does not bind MDM2, but inhibits p53 indirectly through a broad range of mechanisms. Bertout and colleagues showed in two VHL deficient RCC cell lines, that increased HIF-2α level inhibit p53 Ser15 phosphorylation and rescues p53 mediated responses by disrupting cellular redox homeostasis. The inversed effect could be demonstrated by knocking down HIF-2α. Cells, deficient in HIF-2 exhibit increased ATM activity and DNA double strand breaks, as well as increased ROS levels after treatment with ionizing radiation (201). Genes coding for antioxidants such as the *superoxide dismutase 1 and 2 (SOD1, SOD2)*, *glutathione peroxidase 1* (*GPX1*) and *catalase* (*CAT*) in developing embryos and neonates are HIF-2α regulated. However, in RCC cells, HIF-2α expression leads to decreased ROS level due to the regulation of distinct antioxidants enzymes (202). In RCC tumor samples HIF-2α expression correlates with decreased p53 phosphorylation levels and target gene expression, which potentially can contribute to radio resistance of this tumor type. In the same cancer type, HIF-2α supresses p53 expression via growth factor-AKT-MDM2 mediated pathway, additionally to the redox homeostasis. AKT mediates phosphorylation of MDM2, which leads to increased nuclear localisation and enhanced p53 degradation (203).

1.7.3. The role of HIFs in mTOR regulation

High levels of protein synthesis and anabolic metabolism are required for cell division. This is regulated by the serine/threonine kinase mammalian target of rapamycin (mTOR), responds to nutrients and growth factor availability. mTORC1 promotes ribosomal biogenesis, mRNA translation, nutrient transport and inhibits autophagy. In most human tumors, elevated mTORC1 activity can be observed due to oncogene activation, like phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), AKT, and/or the loss of tumorsupressors like phosphatase and tensin homolog (PTEN) or liver kinase B1 (LKB1). The tuberous sclerosis proteins (TSC) TSC1 and TSC2 inhibit mTORC1 activity under environmental stress to limit cell growth (204,205).

During hypoxia mTORC1 is repressed by a variety of mechanisms. In serum depleted HEK 293T cells and rhabdomyosacroma Rho30 cells, decreased ATP levels in severe hypoxia leads to the activation of the AMP activated protein kinase (AMPK), which phosphorylates TSC2 and regulatory-associated

protein of the mammalian target of rapamycin (RAPTOR) (205). It could be shown in MEFs, derived from TSC2 deficient embryos, that HIF-1α induces the expression of the *DNA-damage-inducible transcript 4* (*DDIT4)* gene, encoding for REDD1, which represses mTORC1. The hypoxia-inducible pro-autophagic protein BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) also reduces mTORC1 activity. The cells may benefit from mTORC1 inhibition because of the reduction in protein synthesis and an additional gain of energy due to autophagy (206-208).

Growing evidence suggests that HIF-2α has an opposite effect on mTORC1 promoting cell growth in oxygen deprived cells. FIP200, the focal adhesion protein of 200 kDa, has been identified as a HIF-2α target gene in microarrays. In MEFs, FIP200 is supposed to interact with TSC1 and to disrupt the TSC1/TSC2 complex, which leads to mTORC1 activation. FIP200 promotes also TSC1 proteasomal degradation. HIF-2α dependent AKT stimulation leads to an indirect but selective increase of TORC1 activity (203,209).

HIF-1α and HIF-2α influence oncogenic signalling pathways on different levels, like direct mechanisms via protein-protein interaction or indirectly via gene expression of repressors or activators. Furthermore the HIF and the oncogenic signalling pathways are connected by sharing different transcriptional co-activators. HIF-1α has an inhibitory effect towards growth regulatory systems, which is important for energy conservation due to decreased ATP production in hypoxia and additional nutrient and growth factor availability in the subdomains of solid tumors. MYC is inhibited by HIF-1α. p53 and HIF-1α show a very complex interaction, whereas mTOR1 is inhibited by HIF-1α. In contrast HIF-2α has a pro-proliferative effect. HIF-2α inhibits tumor suppressors, like p53 and increases oncogene activity as shown in the MYC example. Taken together all the effects promote a more aggressive and very resistant cancer phenotype.

1.8. The phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG1)/ C-terminal Src kinase (Csk) binding protein (Cbp)

1.8.1. Discovery and properties of PAG1

Since the early 1990s a heavily tyrosine phosphorylated protein with an approximate size of 80 kDa was repeatedly observed as a major compound of *in vitro* radiolabelled immunoprecipitates of detergent-insoluble membrane microdomains (DRMs) (210-213). This protein was also found in a protein complex containing the Src family of tyrosine kinases (SFK) member Fyn (214). Later the phosphoprotein was identified simultaneously by two different workgroups. Brdicka and coworkers cloned the cDNA of the protein from a human B-cell line and named it PAG1 (215). Kawabushi and coworkers found this protein associated with c-terminal SRC kinase (Csk) in detergent insoluble rafts of the brain and gave it the name Cbp (216).

The *PAG1* gene is found virtually in all vertebrate species with known genome sequence. In humans it is located on chromosome 8, consisting of 9 exons. PAG1 is ubiquitously expressed with highest expression levels in the immune system, lung, heart and placenta (215). PAG1 contains a big 711 bp

containing 5' untranslated region (UTR) and an 8733 bp 3'UTR. The translated protein has a length of 432 amino acids (aa) and is composed of segments as depicted in Fig. 10. The mouse orthologue Pag1 is located on the negative strand of chromosome 3, consisting of 2 exons. The protein product of this gene is only 3 aa shorter, than the human version displaying the same elements.

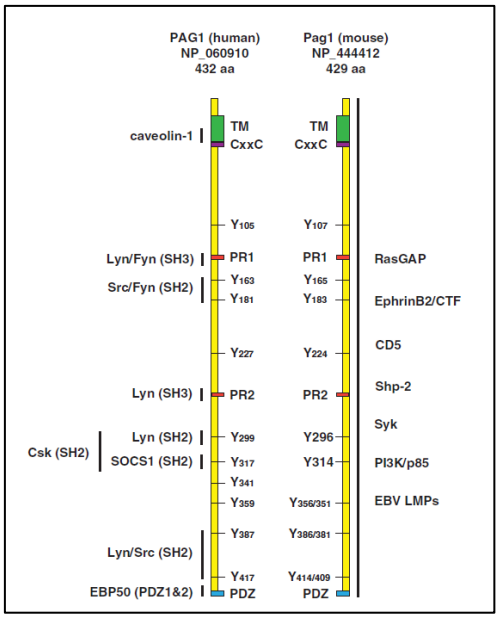

Fig. 10: Overview of human and mouse PAG1 cytoplasmatic domains.
TM (green) shows the transmembrane part, followed by the CxxC motif of palmytoylation. PR1 and PR2 (red) indicate the location of the proline-rich motif and blue the PSD95, Dlg1, ZO-1-binding motif (PDZ) domain at the C-terminus. Vertical lines indicate known binding sites with interaction partners, from (217).

PAG1 is a member of the transmembrane adaptor proteins (TRAPs). The location of most TRAPs to the plasma membrane predestine them to act in the proximal events of various signalling pathways, either as a positive or negative regulator (218). Till today, this family consists of various members with different properties (219), but most of them are located in two different compartments of plasma membrane microdomains, lipid rafts (LR) and tetraspanin-enriched microdomains (TEM), belonging to the DRMs (220). PAG1 is localised in LRs (215). These plasma membrane compartments are important in a variety of cellular processes, like signalling, endocytosis and exocytosis. LR formation is mostly based on certain lipids (cholesterol, sphingolipids), TEMs are organised by protein-protein interactions among members of the tetraspanin family. LRs and TEMs can be distinguished from the rest of the membrane by cell lysis with particular detergents. TEMs are resistant to mild detergents like Brji98, CHAPS and soluble in more stringent one like NP-40, Triton-X-100. LRs are soluble in presence of these detergents (221).

1.8.2. The biochemical properties of PAG1

Based on the amino acid composition the theoretical molecular weight of PAG1 is around 48 kDa, which does not correspond to the observed relative mobility in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). In SDS-PAGE, PAG1 migrates as a fuzzy band around 80-85 kDa, as shown in Fig. 11. This could be caused by numerous modifications, including

phosphorylation, palmytoylation and high content of acidic amino acids (pl=4.2), which can lead to low SDS binding (222).

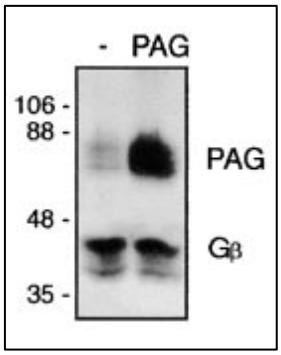

Fig.11: Western Blot analysis of Jurkat cells, transfected with empty (-) or PAG1 overexpression vector (PAG). The fuzzy protein band is located below 88 kDa. β-subunits from heterotrimeric G-protein are used as loading control, from (215).

PAG1 is palmytoylated on its cytoplasmic membrane-proximal cysteine residue, which guides the protein to its essential location in LRs (215,223). The location of PAG1 in LRs is remarkably strong and preserved even in the presence of LR disrupting agents. In contrast to other non-raft associated members of the TRAP family, PAG1 remains on the cell surface, although its interaction partners get internalised (224).

1.8.3. The interaction motifs of PAG1

The cytoplasmic domain of PAG1 contains 10 tyrosine phosphorylation sites, from which nine overlap with a phosphorylation motif preferred by SFKs, as depicted in Fig. 10. This modification is dynamically regulated in response to engagement by specific plasma membrane receptors, like immunoreceptors, growth factor receptors or integrins. PAG1 phosphorylation shows a peak in various cell types within 5-15 min of cell stimulation and returns then slowly back to basal level (224-227).

The only exceptions are T-cells, where PAG1 is dephosphorylated upon T-cell receptor stimulation. In this cell type, PAG1 is mainly phosphorylated by the SFK member Fyn (228). In the T-cell derived cancer cell line Jurkat, also SH2 domain containing leukocyte protein of 76 kDa (SLP-76) can phosphorylate PAG1 at various tyrosine residues (229). In B-cells, mast cells and erythroid cells, PAG1 seems to be mainly phosphorylated by Lyn, another member of the SFKs. As mentioned before in other cell types PAG1 is regulated by more than one SFK member (227,230-232).

1.8.4. The cellular function of PAG1

PAG1 is the major regulator of SFK. They are present in essentially all metazoan cells, where their controlled activation by diverse growth factors, cytokine, adhesion and antigen receptors is critical for generating an appropriate cellular response to external stimuli (233,234). The SFK family of non-receptor tyrosine kinases consists of nine members: Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes and Yrk.

These kinases share a conserved domain structure consisting of consecutive Src homology domains (SH) SH3, SH2 and a tyrosine kinase domain SH1. SFK also contain an SH4 membrane targeting region at their N-terminus, which is always myristoylyated and sometimes palmytoylated. The SH4 domain is also called the “unique” domain of 50-70 aa residues, because it is divergent among all family members (235,236).

An important feature of all SFK is a short C-terminal tail, bearing an auto-inhibitory phosphorylation site (Tyrosine 527 in human Src) (237). For full activation of the protein kinases all SFK require phosphorylation within a segment of the kinase domain. As an example for this family in human Src this site is located at Tyrosine 419 (238). *In vivo,* Src is either phosphorylated on Tyr 419 (active state) or Tyr 527 (inactive state), this phosphorylation of Tyr 527 is carried out by Src specific kinase Csk or its homolog Csk homology kinase (Chk) (239-241).

PAG1 phosphorylation at tyrosine 314 recruits Csk to the membrane into the lipid raft compartment, as it phosphorylates Src at tyrosine 527, which promotes the assembly of the SH2, SH3 and the kinase domains into an auto-inhibitory and closed confirmation. This event initiates a negative feedback-loop, leading to inactivation of Src via Csk. Afterwards, PAG1 phosphorylation returns to basal levels and Csk dissociates from the membrane (243) (Fig.12).

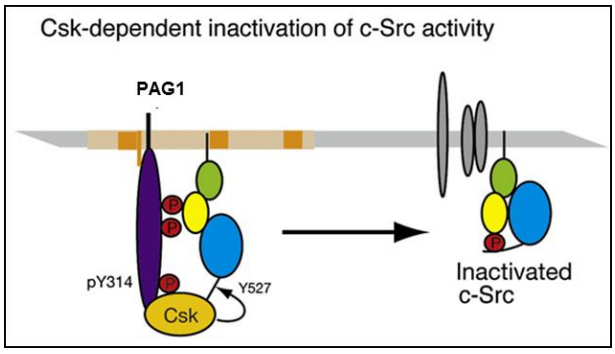

Fig. 12: Scheme depicting Csk dependent SFK regulation, c-Src is shown as an example, adapted from (242).

Another layer of regulation includes the phosphatases PEP and PTP-PEST (244). The phosphatase PEP (human orthologue LYP), only expressed in the hematopoetic system, and the ubiquitously expressed PTP-PEST (PTPN12) were reported to associate constitutively with the SH3 domain of Csk (239,244-246). Therefore they might negatively regulate Src activity together with Csk. Although Csk phosphorylates Tyr 527 of Src, PEP/PTP-PEST would dephosphorylate the Src Tyr 419 residue, leading to a fully inactive Src with closed confirmation. However, *in vivo* data about a regulatory PAG1-Csk-PEP/PTP-PEST axis are still missing. Furthermore a study in resting human T-cells showed that Csk and LYP are uncoupled (247).

1.8.4.1 PAG1 in immune receptor signalling

The activation of lymphocytes requires signals that are mediated through receptors with specificity for antigens (immune receptors). However, these signals alone are insufficient to fully activate these cells;

additional signals are required through co-receptors. A complete activation is only possible when both signals are present and integrated in the cell.

In resting T-cells, PAG1 is phosphorylated and associated with Csk in the LR fraction of the membrane. The basal phosphorylation of PAG1 is maintained by Fyn in these cells (228). Activation of T-cell receptor signalling (TCR) rapidly decreases PAG1 phosphorylation and its association with Csk, moving to the cytosol away from the membrane by association with the adaptor protein G3BP. In primary T-cells and T-cell lines, PAG1 has an inhibitory effect as it was shown in various overexpression studies (215,248-250). This was further supported by the finding that T-cells from transgenic mice overexpressing PAG1 show inhibited TCR activation and inhibition of downstream targets like interleukin-2 (IL-2) production. The overexpression of a mutant variant has a strong dominant negative effect (251). The inhibitory effect of PAG1 in SFK signalling is not only limited to direct TCR signalling, but also influencing the activation via other receptors. Antibody mediated cross-linking of the CD4 receptor leads to reduced TCR signalling via increased association of Csk with PAG1 (252). Besides inhibition via the inhibitory receptor, CD5 interfered with dissociation of Csk from PAG1 and Fyn (253).

PAG1 is also involved in T-cell anergy, caused by strong TCR activation with no other co-stimulus, leading to non-responsive cells. The anergy-promoting complex is composed of hyper-phosphorylated PAG1, Fyn, the multifunctional adaptor protein src-associated in mitosis 68 kDa protein (Sam68) and RasGAP. Overexpressing PAG1 and Fyn in Jurkat cells suppresses Ras activation, leading to the suggestion that PAG1 also regulates Ras activity in these cells (254,255).

Another layer of PAG1 and Csk regulation in T-cells involves intracellular cAMP. Prostaglandin E2 treatment of T-cells increases cAMP levels and inhibits T-cell activation. Under these conditions, the PAG1-Csk complex is stabilised, because of PAG1 Ser-364 phosphorylation by the protein kinase A type I (PKA), due to its activation by cAMP. This interaction leads to a raft- associated supramolecule signalling complex consisting of PKA type I, PAG1, Csk, ERM (Ezrin/Radixin/Moesin)-binding phosphoprotein 50 (EBP50), contributing to T-cell inhibition mediated by cAMP (256-259).

Three different research groups independently generated PAG1 deficient mice to analyse PAG1 function *in vivo*. Two of them analysed the role of PAG1 in T-cell signalling and development. The global PAG1 knockout mouse showed no developmental defects or changes in postnatal growth. Besides a slight increase in the number of thymocytes, no specific phenotype was observed in immune responses to a superantigen, immune cell development or B-or T-cell response in the global knockout PAG1 mouse. In addition a mouse with PAG1 deleted conditionally in CD4-positive T-cells was created, showing any defects in T-cell receptor signalling.

These observations together with analysis of T-cells from bone-marrow transplanted mice, overexpressing a dominant–negative PAG1 mutant, leading to the conclusion that PAG1 itself is dispensable for Csk membrane targeting in T-cells (260-262).

PAG1 phosphorylation levels are increased in B-cells upon B-cell receptor (BCR) stimulation. Treating B-cells with raft disrupting compounds leads to dephosphorylation of PAG1 and reduced intracellular calcium levels (263). This result indicated that the roles of PAG1 and Csk in B-cells are different when

compared to their role in T-cells. In B-cells, the main SFK, Lyn, might phosphorylate mainly the negative signalling regulators, like Siglecs. BCR stimulation leads to increased PAG1 phosporylation and recruitment of Csk to the membrane. Csk is inhibiting Lyn activity and its phosphorylation of Siglecs and therefore activate B-cells (231).
Fyn and Lyn are the two main SFKs involved in BCR signalling. Lyn deficiency results in a hyper-responsive phenotype, which could be explained as mentioned above. Furthermore it was shown that in Lyn deficient cells Fyn activity was increased, leading to no change in PAG1 phosphorylation. This effect could not be explained until now but it is speculated that Fyn and PAG1 are localised in different lipid rafts and no interaction is possible. Other studies suggest that PAG1 has a negative role in BCR signalling rather than maintaining the tonic inhibition of BCR proximal signalling (264).

In high affinity IgE receptor (FcεRI) signalling of mast cells, PAG1 plays a role comparable to B-cells. Shortly after FcεRI activation, SFK hyper-phosphorylate PAG1. This recruits Csk to lipid rafts, leading to SFK inactivation. Thus, PAG1 acts as a genuine feedback-loop inhibitor (232). Furthermore, PAG1 overexpression studies in rat basophils resulted in an effective inhibition of cell activation and degranulation induced by FcεRI signalling (265).

The role of PAG1 in pathogenic processes like anaphylaxis and epilepsy was investigated with two different mouse models. EL mice are an inbred strain and extensively studied as a model of human epilepsy. This strain is anaphylaxis-resistant. ASK mice, originated from a colony of EL mice, are epilepsy-resistant, but an anaphylaxis-prone variant. A comparison of mast cells from both strands revealed a potential influence of PAG1, showing enhanced proliferation and production of tumor necrosis factor α (TNF-α) and IL-2. In mast cells from ASK mice, PAG1 is hypo-phosphorylated and recruits a reduced amount of Csk to the membrane, resulting in hyperactive Fyn and Src, compared to EL derived cells (266).
Mast cells from mice lacking Hck, a SFK family member, show the opposite effect, with increased Lyn activity, leading to an increase in PAG1 phosphorylation and inhibition of FcεRI signalling (267).

The role of PAG1 in immune cell receptor signalling, as referred above, is very broad and complex. Although the role of PAG1 in T-cell receptor signalling could not be confirmed *in vivo*, an influence in this signalling pathway cannot be completely excluded. The role of BCR still lacks a translation *in vivo*, whereas the role in FcεRI signalling was found exclusively in mice.

1.8.4.2. PAG1 in non-immune cell regulation

In fibroblasts, PAG1 remains hypo-phosphorylated until the cells are treated with growth factors like endothelial growth factor (EGF) or platelet-derived growth factor (PDGF) (226,268). PAG1 phosphorylation status also changes when the cells adhere to the extracellular matrix (269). In this cell type, expression of a dominant-negative mutant or knockdown of PAG1 impairs recruitment of Csk to lipid rafts. Overexpression of PAG1 in fibroblasts can supress EGF-induced downstream activation of ERK and AKT phosphorylation, cell transformation and impaired colony formation in soft agar (226).

The dephosphorylation of PAG1 is mediated by the protein tyrosine phosphatase (PTP) SHP-2 required for growth factor induced SFK activation. During stimulation, Src homology region 2-containing protein tyrosine phosphatase-2 (Shp-2) opposes the SFK action by dephosphorylation of PAG1. In Shp-2 deficient fibroblasts, PAG1 is hyperphosphorylated, leading to reduction in SFK activity (270). The phosphorylation status of PAG1 in these cells can also be influenced by receptor protein tyrosine phosphatase-α (RPTP-α), indirectly via enhanced Fyn activity (271).

In fibroblasts, PAG1 is also involved in the integrin signalling. Activation of integrins at focal adhesion sites control fibroblast adhesion to the extracellular matrix and prevents cell migration. PAG1 is involved in the inside-out β1 integrin signalling via dioxin receptor/aryl hydrocarbon receptor AhR. PAG1 contains a XRE in its promoter region. The AhR/ARNT heterodimer binds to XREs, downregulating PAG1 transcription. Fibroblasts of AhR-knockout mice show increased β1 intergrin activity and impaired migration, due to PAG1 overexpression. These increased levels of PAG1 target more Csk to the membrane and negatively regulate Src and its downstream targets focal adhesion kinase (FAK) and caveolin (272).

The differentiation of erythroid cells is stimulated by Epo. Epo binds to its receptor EpoR, which after ligand binding dimerizes and activates the Janus kinase 2 (JAK2) by trans-phosphorylation (273,274). JAK2 itself also phosphorylates the Epo receptor on different tyrosine residues within the cytoplasmatic domain. This serves as a docking site for numerous signalling molecules, like signal transducer and activator of transcription 5 (STAT5), which associates with the Epo receptor and is phosphorylated by JAK2, leading to nuclear translocation and activation of gene transcription (275-277). Other pathways involved in EpoR signalling include Ras/Raf/MAP kinase and PI3-kinase/AKT (278,279). The downregulation of these pathways is performed in two steps. In the first step, the phosphatase SHP-1 is docking on the phosphorylated tyrosine residues of Epo receptor and dephosphorylates them with its SH2 domain (280). Several members of the suppressor of cytokine signalling (SOCS) family are actived upon Epo receptor stimulation. These proteins inhibit JAK2 activity and compete with STAT5 for JAK2 binding. In addition the association of SOCS proteins with elonging B/C leads to ubiquitination and proteasomal degradation of JAK2 (281-283). The relevant SFK member involved in EpoR signalling is Lyn, required for STAT5 phosphorylation. Lyn is involved in Crk-like adaptor protein (CrkL) activation after EpoR stimulation (284). In the first step of EpoR inactivation, Lyn activation is increased, resulting in the phosphorylation of PAG1 on several tyrosine residues including Tyr 314, Tyr 381, Tyr 409. Phosphorylation of the latter two residues leads to a closer association between PAG1 and Lyn, mediated by the SH2 domain of Lyn. PAG1 Tyr 314 phosphorylation leads to recruitment of Csk/ tyrosine-protein kinase (Ctk) to the complex, which inactivates Lyn by phosphorylation of Tyr 508. This occurs 10-30 min after EpoR stimulation. The second step, occurs several hours after stimulation. Lyn and JAK2/STAT pathways induce SOCS. SOCS then associates with PAG1 and promotes the ubiquitination and proteasomal degradation of Lyn (227). PAG1 overexpression in J2E erythroid cells significantly reduced colony formation, Epo-induced haemoglobin synthesis and phosphorylation of several proteins (285).

In the kidney, podocytes are highly specialized cells involved in the first step of renal blood filtration. In these cells, PAG1 is one of the key proteins in angiotensin II-induced cell damage. The major structural protein of the split diaphragm is nephrin, a member of the immunoglobulin family of cell adhesion molecules. The split diaphragm of the glomerular podocytes functions as an ultrafilter to exclude plasma macromolecules and blood albumin from the primary urine (286). Nephrin is regulated by Fyn phosphorylation and has an anti-apoptotic function. Angiotensin II treated podocytes overproduce Csk, which forms a plasma membrane complex with hyperphosphorylated PAG1, inhibiting Fyn function. This leads to reduced nephrin phosphorylation and inhibits its anti-apoptotic function (287).

The PAG1-Csk scaffold also plays a role in bone resorption. Effective osteoclastic bone resorption requires high levels of active Src. Mice deficient in Src develop severe osteopetrosis (288). In osteoclasts PAG1 recruits Csk to the membrane and interferes with Src activity. PAG1 expression is downregulated by the receptor activator of NF-kappaB ligand (RANKL) signalling pathway. Ectopic expression of PAG1 in osteoclasts leads to inhibition of Src activation and prevents bone resorption (289).

PAG1 also functions as a Csk adaptor protein in rat brain (216). An additional site of tyrosine phosphorylation Tyr 105 could be identified in rat brain tissue in addition to the essential Tyr 314 residue (290). A PAG1 knockout mouse was created to analyse the influence of PAG1 in brain maturation. The PAG1 knockout mice did not show any neuronal phenotype. Different developmental stages of PAG1 knockout mice were analysed for the impact of PAG1 as scaffold protein for Csk and its brain homologue Chk. In postnatal (P1) brain of PAG1 knockout mice, a 50% increase of Fyn and Src and a 40% decrease of inhibitory tyrosine phosphorylation could be observed. In the juvenile brain (P42) Csk is not only bound to PAG1 anymore, suggesting that PAG1 knockout mice develop a compensatory mechanism for the lack of PAG1, maybe by postsynaptic density 93 (PSD93), another transmembrane adaptor. Compensatory mechanisms would explain more generally the lack of phenotype of PAG1 deficient mice (291).

The PAG1-Lyn complex has been shown to be associated with ganglioside GD3 and to be enriched in the growth cone of developing neurons, suggesting a potential role in neuronal differentiation (292).

In rat neurons PAG1 in involved in the ephrinB2 signalling as negative regulator of Src. After EphB2 receptor binding, ephrinB2 ligand is cleaved by the PS1/γ-secretase complex. The cleavage product, the ephrinB2/CTF2 fragment, associates with PAG1 and promotes its dephosphorylation, leading to Csk release and Src activation. Active Src phosphorylates ephrinB2 ligand. Short interference RNA mediated PAG1 downregulation leads to ephrinB2 ligand phosphorylation. Several PS1 mutants found in familiar Alzheimer's disease (FAD) inhibit the cleavage, the PAG1 dependent phosphorylation of ephrinB2. These findings suggest a potential role of PAG1 in Alzheimer disease (293).

Conversely, laminin substrates were found to promote the transition of oligodendrocyte progenitors to newly formed oligodendrocytes. Laminin-enhanced differentiation was Src family kinase-dependent and resulted in the activation of the Src family kinase Fyn. In oligodendrocytes the PAG1-Csk scaffold negatively regulates Fyn. Cells from laminin deficient mice express double amount of PAG1 and Csk, supressing Fyn activity, leading to a delay of development due to lack of laminin, suggesting that Fyn is regulated by a laminin-PAG1-Csk scaffold (294).

The regulatory scaffold SFKs- PAG1-Csk was found to influence a set of different pathways summarised in Table 5.

Pathway	Cell type	Effect of PAG1-Csk scaffold
TCR signalling	Primary T-cells, T-cell lines	Inhibition
T-Cell anergy	Jurkat	Inhibition
BCR signalling	B-lymphocytes	Activation
FcεRI signalling	Mast cells	Inhibition
EGF/ PDGF signalling	Fibroblasts	Inhibition
EpoR signalling	J2E erythroid cells	Inhibition
Angiotensin II signalling	Podocytes	Inhibition
RANKL signalling	Osteoclasts	Inhibition
Brain maturation	Global Pag1 knockout mouse	No neuronal phenotype
EphrinB2 signalling	Fibroblasts from PS1, Pag1 knockout mice	Activation
Laminin-enhanced differentiation	Oligodendrocytes	Inhibition

Table 5: Summary of all pathways influenced by the PAG1-Csk scaffold in different cell lines or cell types.

1.8.4.3. Negative SFK regulation independent of Csk

PAG1 is able to negatively regulate SFKs without Csk recruitment. PAG1 overexpression in mouse embryonic fibroblasts strongly inhibits PDGF receptor (PDGFR)-Src signalling independent of Csk. The N-terminal part of PAG1 (amino acid residues 1-97) probably displaces PDGFR from the caveolae by sialidase Neu3 and ganglioside GM1 binding. This is only possible if the caveolin-1 binding site of PAG1 is intact (295). PAG1 is able via phosphorylated Tyr residues 165 and 183 (in mouse Tyr 381 and in rat Tyr 409) to directly bind Src and maintain an inactive state inside the lipid raft fraction (243).

1.8.4.4. PAG1 as a linker between lipid rafts and cytoskeleton

As depicted in Fig. 10, PAG1 contains a C-terminal PDZ binding motif that can be recognized by both PDZ domains of the scaffolding protein EBP50/Na+/H+ exchanger regulatory factor (NHE-RF). Thus, PAG1 is part of the EBP50-ezrin complex which is the major anchor of lipid rafts to the cytoskeleton (296). *In vivo*, only a minor fraction of PAG1 is bound by EBP50. This cytoskeleton link is reversible (297). Upon B- or T-cell stimulation, the association between PAG1 and EBP50 is lost and the EBP50 presence in lipid rafts is decreased (298,299). A potential role for such interactions could be a PAG1 dependent regulation of lipid raft dynamics, which is important for immune receptor activation. Overexpression of PAG1 in Jurkat T-cell and Raji B-cells revealed markedly increased surface mobility of the lipid raft marker GM1, and prevented formation of an immunological synapse in a EBP50 dependent manner (298). Raft-displaced palmytoylation defective PAG1 is unable to inhibit proximal T-cell signalling, although it is still interacting with the Fyn-Csk-EBP50 complex (223).
Another potential reason for PAG1-EBP50 interaction could be the targeting of PKAI. PKAI is activated by cAMP binding and stimulates Csk activity via phosphorylation of Csk Ser-364, contributing to the before mentioned TCR signalling role (300).

The PAG1-EBP50 interaction could also be important in a process called “transient anchorage”. Glycosylphosphatidylinositol (GPI)-anchored proteins are important compound of lipid rafts. These proteins are able to transmit signals into the inside of the cell, although they lack any cytoplasmic domain, but potentially indirectly associate with raft proteins including TRAPs. In transient anchorage, these GPI-proteins stop for milliseconds, suggesting a short term interaction with the cytoskeleton (301,302). In rodent T-cells the GPI-anchorage protein Thy-1 cell surface antigen (Thy1) interacts with PAG1 at the plasma membrane and is involved in transient anchorage (303).

These findings suggest a role in cytoskeleton modification, independent of the SFK and Csk regulation.

1.9. The role of PAG1 in cancer

In cancer, PAG1 is either up or downregulated in tumor cells. The downregulation of PAG1 leads to hyperactive SFKs and to cell transformation. Of note, SFK genes have not been found to be mutated in cancer, but acquire oncogenic features by dysregulation. However, also cancers with up-regulated PAG1 protein levels are reported. These findings suggest that PAG1 itself has an oncogenic role in cancers.

1.9.1. Mechanisms involved in downregulation of PAG1 in cancer cells

PAG1 downregulation was first addressed in Csk deficient MEFs. Following activation of the oncogenic ERK-MAPK/PI3K pathway the expression of PAG1 was reduced in $Csk^{-/-}$ MEFs. Overexpression of SRC and Ras and other members of this pathway led to the same effect on PAG1 mRNA and protein levels. Stimulation of ERK-MAPK/PI3K pathway by EGF treatment showed a dose dependent reduction of PAG1 mRNA after 10 hours and of protein levels after 48 hours. Since no involvement of a specific transcription factor could be shown, bisulfite sequencing was performed in a larger number of cell lines, including MCF-7, HT19 and A549. All cells showed a hypomethylated *PAG1* promoter region. Using short interfence RNA experiments, the histone deactylases HDAC1 and 2 were found to be involved in the epigenetic regulation of the PAG1 promoter region, leading to reduced PAG1 mRNA expression Src mediated HDAC1/2 histone deacetylation and PRC2 are involved in this mechanism (304). The aforementioned regulation of PAG1 by AhR also contributes to the downregulation of PAG1 in cancer cells (272).

In fibroblasts uncontrolled Src activity can lead to changes in cell morphology and anchorage-independent growth, two features of cell transformation and tumorigenesis. These effects are even stronger in Csk deficient cells, where PAG1 is constitutively hyperphosphorylated. Reconstitution of Csk expression leads to PAG dephosphorylation. In cells lacking PAG1, cell spreading is enhanced. These observations point to the conclusion that PAG1 acts as a sensor of SFK activity in these cells, likely by mediating SFK feedback inhibition due to recruitment of Csk to the lipid raft fraction (269). Endogenous Src levels in these cells do not induce transformation. However, exogenously increased levels of Src can overcome this block. In such cells, PAG1 is strongly down-regulated due to unknown

mechanisms. Reexpression of PAG1 in these cells, even without Csk, can block transformation, demonstrating that PAG1 is able to control Src activity directly. Src, a non-lipid raft SFK can phosphorylate non-raft targets. Being sequestered to lipid rafts by PAG1, Src loses its oncogenic properties (243,305). The oncogenic potential of SFKs is apparently dependent on their membrane microenvironment. SFKs, which are also distributed over non-raft fraction, like Src and Blk, can transform $Csk^{-/-}$ fibroblasts, in contrast to raft localised SFK members, like Fyn and Lyn, which cannot transform these cells. PAG1, also raft-localised has the potential to interact/associate with all active SFKs. This finding is an unique feature of PAG1 among all TRAP members, suggesting a role for PAG1 in SFK membrane homeostasis and controlling their transforming potential in fibroblasts (306).

In other transformed cell types, SFK regulation is done by the PAG1-Csk scaffold. In various colorectal cancer cell lines the expression of PAG1 is reduced. Reexpression of PAG1 into these cells led to increased membrane localisation of Csk and to reduced invasiveness. Csk knockdown or treatment with the Csk inhibitor ASN2324598 reduced this effect, showing that PAG is essential for Csk recruitment and hence for limiting SFK activity (307). A clear correlation between PAG1 downregulation and Src hyperactivity could be shown in non-small lung cancer cells. Expression of PAG1 in this cell type leads to recruitment of Csk to the membrane, leading to a reduction in Src activity and lost invasion *in vitro* and *in vivo* (308). A downregulation of PAG1 mRNA and protein levels was also reported in esophageal carcinoma samples (309).

The downregulation of PAG1 is one of the strategies of deregulated tumor cells. This strategy has a good explanation, since PAG1 was repeatedly reported to negatively regulate SFK. The removal of PAG1 as regulator might lead to hyper-activation of SFK and cell transformation. An overview of described pathways of PAG1 downregulation is depicted in Fig. 13.

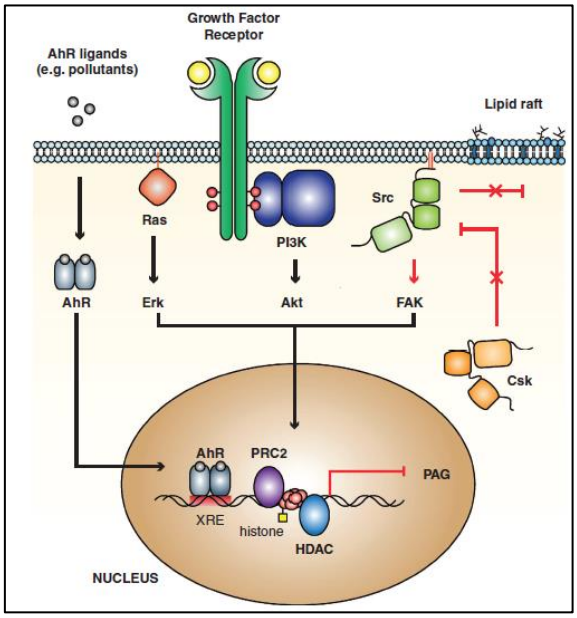

Fig. 13: Mechanism of downmodulation of PAG1 in cancer cells, from (216).

1.9.2. Cancers with up-regulated PAG1

PAG1 was intensively studied in several human lymphoma of non-Hodgkin type lymphomas. Expression of PAG1 is strongly increased in most germinal center-like diffuse large B-cell lymphomas. In contrast, PAG1 is barely detectable in mantle cell lymphoma and chronic lymphocytic leukemias. In these cases, PAG1 could serve as a positive marker for germinal center-like diffuse large B-cell lymphomas, in the germinal center of secondary lymphatic follicles, and for follicular malignant lymphomas to distinguish them from mantle cell lymphomas (310).
In the TEL/AML1 subgroup of childhood B-cell $CD19^+$ and $CD10^+$ of acute lymphoblastic leukemias, PAG1 expression on mRNA and protein level is strikingly higher than in other subgroups of this cancer type and the non-malignant controls (311). These observations suggest a role for PAG1 as prognostic marker in these cancer types.
PAG1 is also up-regulated in more than 70% of renal cell carcinomas and their corresponding cell lines. *In vitro*, PAG1 knockdowns in the clear cell renal cell carcinoma line 786-O, lowered proliferation, cell motility, invasion and anchorage independent growth. Furthermore, tumor formation in nude mice was abolished. The activation levels of the PAG1 target c-Src and its downstream targets ERK1/2 and AKT remained unchanged in the PAG1 knock-down cells. Since also changes in cytoskeleton with increased intracellular F-actin levels were observed, analysis of ras homolog family member A (RhoA), cell division cycle 42(Cdc42) and ras-related C3 botulinum toxin substrate 1 (Rac1), molecules involved in intracellular cytoskeleton regulation, were performed. PAG1 knock-downs showed reduced levels of active RhoA, whereas Cdc42 and Rac1 levels remained unchanged. Overexpression of PAG1 wild type protein, leads to increased activation of RhoA and to enhanced migration, whereas a PDZ deficient PAG1 construct failed to induce the same effects, supporting the hypothesis, that the PDZ domain of PAG1 is involved in cytoskeleton regulation (312).

In laryngeal carcinoma cells, PAG1 is increased in a radiotherapy-resistant subtype. Knockdown of PAG1 in these cells led to increased radiotherapy-mediated cell death (313). In human embryonic kidney cancer HEK293 cells and in the triple negative breast cancer cell line MDA-MB-231, high PAG1 levels could be identified (243). The increased expression levels might be explained by a formation of a raft-resistant PAG1-based signallosome. PAG1 is constantly phosphorylated, but uncoupled from Csk. In different lymphoma cell lines, this PAG1-based signallosome contains constant levels of phosphorylated PAG1, Lyn, Lyn-bound STAT3 and variable levels of Syk and PI3K (Fig.14).

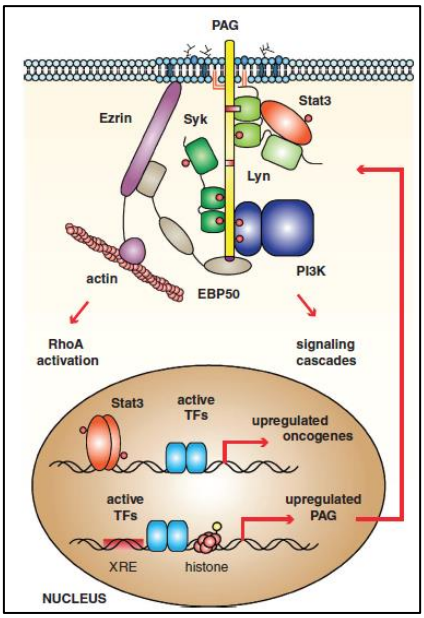

Fig.14: Mechanism of PAG1 up-regulation in cancer cells, from (216)

In vitro studies, performed in the mantle-cell lymphoma cell line Jeko-1 showed that the signallosome complex contains only weak levels of PI3K. In the Burkitt-derived lymphoma cell line (Raji) only Syk is part of the complex. These cells are Eppstein-Barr Virus transformed and expressing the latent membrane proteins of this virus, these viral proteins may associate with PAG1 at the membrane and block PI3K kinase binding under these conditions, Syk can be directly activated by Lyn, independent of BCR signalling. Functional disruption of the oncogenic signallosome by Lyn inhibition or PAG1 knockdown in non-Hodgkin B lymphomas resulted in increased cell death (314,315).

The up-regulation of PAG1 displays the second strategy of deregulated cancer cells. Although some mechanisms, as depicted in Fig.14, are known, the overall picture contributing the up-regulation of PAG1 in cancer cells remains elusive.

1.10. References

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2. Aims of the thesis

PAG1 is known to be a ubiquitously expressed gene, with potential functions in different physiologically relevant processes. In this thesis we identified *PAG1* as a novel hypoxia inducible gene.

2.1. Aims:

- Characterisation of the *PAG1* hypoxic response *in vitro* and *in vivo.*
- Dissection of the molecular mechanism involved in hypoxic *PAG1* regulation: identification of the HRE(s).
- Investigation of the physiological meaning of hypoxic PAG1 induction for Src.

Destruction of a distal hypoxia response element abolishes *trans*-activation of the *PAG1* gene mediated by HIF-independent chromatin looping

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ABSTRACT

A crucial step in the cellular adaptation to oxygen deficiency is the binding of hypoxia-inducible factors (HIFs) to hypoxia response elements (HREs) of oxygen-regulated genes. Genome-wide HIF-1α/2α/β DNA-binding studies revealed that the majority of HREs reside distant to the promoter regions, but the function of these distal HREs has only been marginally studied in the genomic context. We used chromatin immunoprecipitation (ChIP), gene editing (TALEN) and chromosome conformation capture (3C) to localize and functionally characterize a 82 kb upstream HRE that solely drives oxygen-regulated expression of the newly identified HIF target gene *PAG1*. PAG1, a transmembrane adaptor protein involved in Src signalling, was hypoxically induced in various cell lines and mouse tissues. ChIP and reporter gene assays demonstrated that the -82 kb HRE regulates *PAG1*, but not an equally distant gene further upstream, by direct interaction with HIF. Ablation of the consensus HRE motif abolished the hypoxic induction of *PAG1* but not general oxygen signalling. 3C assays revealed that the -82 kb HRE physically associates with the *PAG1* promoter region, independent of HIF-DNA interaction. These results demonstrate a constitutive interaction between the -82 kb HRE and the *PAG1* promoter, suggesting a physiologically important rapid response to hypoxia.

INTRODUCTION

Hypoxia, defined as mismatch between oxygen supply and consumption, plays a crucial role in many physiological and pathophysiological conditions, such as embryonic development, adaptation to high-altitude, wound healing, inflammation, cardiovascular diseases and cancer. Hypoxia-inducible factors (HIFs) are the master regulators of cellular adaptation to hypoxia (1). HIFs are heterodimeric transcription factors consisting of a labile oxygen-regulated α-subunit and a constitutively expressed β-subunit. Under normoxic conditions, HIFα subunits are hydroxylated by the oxygen-sensing prolyl-4-hydroxylase domain (PHD) enzymes and the factor inhibiting HIF (FIH) on two prolyl and one asparagine residue, respectively. HIFα prolyl-4-hydroxylation leads to the binding of the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) followed by polyubiquitylation and proteasomal destruction (2,3). Under hypoxic conditions HIFα subunits remain stable, translocate to the nucleus, heterodimerize with HIFβ and transcriptionally activate numerous target genes involved in the adaptation to hypoxia. Therefore, HIF complexes interact directly with the consensus core motif 5'-RCGTG-3', the actual HIF-binding site (HBS) within the hypoxia response element (HRE) (4).

Pan-genomic studies combining techniques to assess transcriptional activity and protein-DNA interaction functionally identified HREs within the promoter regions as well as HREs far away from transcriptional start sites (TSSs) (5-10). Interestingly, 60% of all HIF-1α and 80% of all HIF-2α interactions with HREs locate to distal sites more than 2.5 kb outside of the TSS (7). This finding contrasts with the majority of published single-gene studies which show a certain bias (approx. 76% HREs are within 2.5 kb upstream of the TSSs) towards proximal HREs (4). Furthermore, these pan-genomic analyses also revealed a considerable variability between various cell types, especially for distal HREs (11). Only a few distant HREs have been investigated so far, including those regulating the genes *IGFBP3* (-57 kb upstream of the TSS) (12), *CCND1* (-220 kb) (8), *SLC2A3* (-35 kb) (9) and the putative 5' kidney-inducible element of the *EPO* gene (-9.2 kb) (13). How these distant HREs interact with the promoter regions, whether this interaction is dependent on the presence of HIFs (or even the HBS itself) and how this promoter-enhancer interaction varies between different cell types remains generally unknown.

By using gene arrays to assess the transcriptional profile of HeLa cells under normoxic and hypoxic conditions, we identified the lipid raft phosphoprotein associated with glycosphingolipid enriched microdomains (*PAG1*) as a novel hypoxia-inducible gene. PAG1 is an ubiquitously expressed transmembrane adaptor protein that binds the protein tyrosine kinase csk and is hence also known as csk-binding protein (Cbp) (14,15). We identified a distal HRE located -82 kb upstream of the *PAG1* TSS and used the TALEN (transcription activator-like effector-based nuclease) technique to specifically target the HBS of this remote HRE, resulting in functional ablation of the hypoxia-inducibility of *PAG1* gene transcription. Chromosome conformation capture (3C) assays were employed to assess the interaction between this HRE and the PAG1 promoter region under normoxic, hypoxic, HIF-depleted and HBS-destructed conditions.

MATERIALS AND METHODS

Plasmid constructs

A reporter gene plasmid containing 1014 bp of the *PAG1* promoter was obtained from BioCat (Heidelberg, Germany). The *PAG1* -82 kb HRE region was amplified by Phusion polymerase-based PCR (Thermo Fisher Scientific, Waltham, MA, USA) using HeLa or MDA-MB-231 genomic DNA and the primers (Microsynth, Balgach, Switzerland) listed in Supplementary Table 1. Following restriction digestion with BglII (Thermo Fisher Scientific) fragments of 317 bp and 2 kb were cloned into the pGL3prom vector (Promega, Madison, WI, USA) upstream of the SV40-driven firefly luciferase gene. The HBS was inactivated by site-directed mutagenesis (5'-CGTG-3' to 5'-ATAA-3'; Stratagene, La Jolla, CA, USA). All constructs were verified by sequencing (Microsynth). pH3SVL (16), PHD2 (17), PAI-1 and CAIX (18) reporter gene constructs were described previously.

Cell culture and transfection

HeLa, U2OS, MCF-7, Hep3B, MDA-MB-231, 786-0, 786-VHL, TK188 and TZ-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA). All media were supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Grand Island, NY, USA) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Sigma). HK2 were cultured in DMEM/F12 (Sigma) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 36 ng/ml hydrocortisone and ITS solution (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium; Roche, Basel, Switzerland). Hypoxia experiments were performed at 0.2% or 0.5% O_2 and 5% CO_2 in a gas-controlled workstation (Invivo$_2$ 400; Baker Ruskinn, Bridgend, South Wales, UK). Cells were transfected using polyethylenimine (Polysciences, Warrington, PA, USA) as described previously (19).

RNA isolation and analysis
Total RNA was isolated from cultured cells using the guanidine isothiocyanate method as described before (17). RNA from mice exposed to inspiratory hypoxia was obtained as described elsewhere (20). Specific mRNAs were quantified by reverse transcription followed by quantitative PCR (RT-qPCR) using a MX3000P light cycler (Agilent, Santa Clara, CA, USA) as described previously (17). For gene array analysis, total RNA was extracted from HeLa cells cultured under normoxic or hypoxic (16 hours, 0.2% O_2) conditions with RNeasy (Qiagen, Venlo, Netherlands). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer. Two-colour labelled samples were hybridized to an Agilent whole human genome 4x44K oligonucleotide microarray slide. For each condition two biological replicates were used.

Analysis of human renal biopsies
Human renal biopsy specimens and Affymetrix microarray expression data (HG-U133 Plus2.0 Array) were procured within the framework of the European Renal cDNA Bank - Kröner-Fresenius Biopsy Bank (21). Diagnostic renal biopsies were obtained from patients after informed consent and with approval of the local ethics committees. Following renal biopsy, the tissue was transferred to RNase inhibitor and microdissected into glomerular (Glom) and tubulointerstitial (Tub) compartments. The microarray expression data used in this study came from individual patients with diabetic nephropathy (DN, Glom (n=7), Tub (n=7), focal segmental glomerulosclerosis (FSGS, Glom (n=16), Tub (n=7)), rapidly progressive glomerulonephritis (RPGN, Glom (n=23), Tub (n=21)) as well as pre-transplant biopsies from living renal allograft donors as controls (LD, Glom (n=18), Tub (n=18)). Total RNA was isolated from microdissected glomeruli and tubulointerstitium, reverse transcribed, and linearly amplified according to a protocol previously reported (22). Fragmentation, hybridization, staining, and imaging were performed according to the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). For microarray analysis Robust Multichip Analysis (RMA) was performed. Following normalized RMA, Significance Analysis of Microarrays (SAM) was conducted using a q-value of less than 5% to identify genes that were differently regulated between the analyzed groups (23). RT-qPCR validation of renal biopsies was performed as reported earlier (21). Pre-developed TaqMan reagents were used for human PAG1 (NM_018440.3, Hs00179693_m1) and transcript levels were normalized to 18S rRNA levels (Applied Biosystems, Waltham, MA, USA).

RNA interference
Expression vectors encoding short hairpin RNA (shRNA) sequences targeting human HIF-1α (Sigma number TRCN0015301048s1c1), HIF-2α (TRCN0014361694s1c1), PAG1 (TRCN00000123272) and a non-targeting control shRNA (shCtrl) under the control of a U6 promoter in a pKLO.1 puromycin resistance vector were purchased from Sigma. Alternatively, the puromycin selection cassette has been replaced by a hygromycin cassette. Lentiviral particles were produced in HEK293T cells using the Vira-Power lentiviral expression vector system according to the manufacturer's instructions (Invitrogen). HeLa cells were infected with lentiviral particles containing shHIF-1α, shHIF-2α or shCtrl RNA, followed by selection with 10 μg/ml puromycin to create shHIF-1α, shHIF-2α and shCtrl cells. To generate shHIF-1α/2α double knockdown cells, shHIF-2α cells were infected with lentiviral particles containing a hygromycin-resistant shHIF-1α expression vector, followed by selection with 10 μg/ml puromycin and 1 mg/ml hygromycin. Single and double shHIFα MCF-7 as well as shHIF-2α 786-0 cells have been described previously (24-26). Hep3B cells were infected with shPAG1 lentiviral particles followed by selection with puromycin (1.5 μg/ml).

Protein extraction and analysis
Cells were washed twice with ice-cold PBS, and soluble proteins were extracted with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 400 mM NaCl, 0.1% Nonidet P-40, freshly supplemented with 1x protease inhibitor cocktail, 1 mM NaF and 10 mM Na_3VO_4 (all obtained from Sigma). Insoluble proteins were further extracted with 1% SDS, 5 mM Tris-HCl pH 6.8, 1% Triton X-100 (Sigma), supplemented as above, by ultrasonication (Cell Disruptor B15, Branson, Danbury, CT, USA). Protein concentration was estimated using the BCA method (Thermo Fisher Scientific) and 60-100 μg total protein were subjected to immunoblot analysis using the following mouse monoclonal (mAb) or polyclonal antibodies (pAb): mAb anti-hHIF-1α (clone 54; BD Transduction Laboratories, San Jose, CA, USA), rabbit pAb anti-hHIF-2α/EPAS1 (Abnova, Taipei City, Taiwan), mAb anti-hPAG1 (clone MEM-255; Abnova). For immunoprecipitation, proteins were extracted using ODG buffer (250 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 60 mM octyl β-D-glucopyranoside, freshly supplemented as above). After pre-clearing, extracted protein (800 μg) was mixed with 8 μl anti-PAG1 mAb (Exbio, Vestec, Czech Republic). Following incubation overnight at 4°C, the resin was washed

three times with 1 ml of washing buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl), resuspended in loading buffer, boiled and subjected to SDS-PAGE. Immunoblotting was performed using a pan-tyrosine phosphorylation mAb (Merk Millipore, Darmstadt, Germany) or a rabbit anti-PAG1 pAb (Santa Cruz, Dallas, Texas, USA). Primary antibodies were detected with goat-anti-mouse or goat-anti-rabbit HRP-coupled pAb (Santa Cruz). Chemiluminescence detection was performed using Supersignal West Dura (Thermo Fisher Scientific) and recorded with a CCD camera (LAS-4000; GE Healthcare, Chalfont, St. Giles, UK) followed by quantification with Quantity One software (Bio-Rad, Hercules, CA, USA). Phosphorylation of 46 specific phosphorylation sites was analysed using the Proteome Profiler Human Phospho-Kinase Array Kit (ARY003B), according to the manufacturer's instructions (R&D Systems, Abingdon, UK). Briefly, Hep3B cells were grown for 24 hours, rinsed with ice-cold PBS and resuspended in lysis buffer. Antibody arrays were incubated with cell lysates (350 μg) at 4°C overnight. Signals were detected and quantified as above.

Reporter gene assays
Cells were co-transfected with 10 ng pRLSV40 *Renilla* control luciferase plasmid (Promega) together with either 3 μg (HeLa) or 400 ng (MCF-7) firefly luciferase plasmid or a combination of each 1.5 μg (HeLa) or 200 ng (MCF-7) firefly luciferase construct and overexpression or empty vectors. After 16 hours cells were exposed to either 21% O_2 or 0.2% O_2 for the indicated time points. Cells were lysed in 50 μl Passive Lysis Buffer (Promega) and luciferase activities were determined in triplicates using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Reporter gene activities were expressed as ratios between firefly and *Renilla* luciferase activities.

Gene editing
Pairwise TALEN plasmids to target the -82 kb HBS within the *PAG1* HRE were obtained from Labomics (Nivelles, Belgium). HeLa and MCF-7 cells were transfected with each 1.5 μg TALEN vector and either 500 ng of an EGFP expression vector or 10 to 30 ng linearised pBabe-puromycin vector. After 16 hours, the cells transfected with EGFP were cloned by limited dilution in 96-well plates and EGFP expressing cells were further expanded. After 72 hours, the puromycin co-transfected cells were selected with 10 μg/ml (HeLa) or 2 μg/ml (MCF-7) puromycin. Resistant cells were cloned by limited dilution in 96-well plates. Following expansion of the cloned cells, genomic DNA was isolated and the -82 kb *PAG1* region amplified by PCR as described above. PCR products were either digested with BsaAI (New England Biolabs, Ipswich, MA, USA) and analyzed by 1% NuSieve agarose gel electrophoresis (Lonza, Basel, Switzerland) or cloned into a plasmid vector and sequenced (Microsynth).

Chromatin immunoprecipitation (ChIP) and chromosome conformation capture (3C)
ChIP experiments using HIF-1α, HIF-2α, HIFβ, histone modifications or p300 occupancy were performed as described previously (7,8). Samples were analysed by qPCR using the primers listed in Supplementary Table 2. All values were displayed as fold enrichment over the negative control from 3 independent experiments. 3C experiments were performed as described (27) with some modifications. Cells were grown under normoxic or hypoxic (0.2% O_2, 24 hours) conditions and cross-linked with ethylene glycol-bis(succinimidyl succinate) (Thermo Fisher Scientific) for 45 minutes at room temperature. Nuclei were fixed with 1% formaldehyde for 10 minutes, quenched with 1% glycine and digested with EcoRI. Fragments were diluted in ligation buffer and ligation was performed for 4 hours at 16°C using T4 ligase. Cross-linking was reversed at 65°C overnight and DNA was extensively purified before PCR amplification using the primers listed in Supplementary Table 3. An equimolar mixture of two BAC clones (RP11-624P8 and RP11-21K19), covering the entire region of interest, was used to create an artificial library of ligation product to control for PCR efficiency. The *EEF1G* locus was used to control for crosslinking efficiency between different experiments. PCR products were run on agarose gels, recorded by AlphaImager (Alpha Innotech) and quantified with ImageQuant software. All data were normalized to the BAC ligation products and the *EEF1G* control.

Statistical analysis
If not otherwise indicated, results are presented as mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software).

RESULTS

Hypoxia induces PAG1 expression

Gene array analysis of HeLa cells cultured under normoxic or hypoxic conditions revealed a number of previously unreported oxygen-regulated genes (Supplementary Table 4). mRNA levels of several well-established HIF target genes, including *CA9*, *NDRG1*, *LOX* and *EGLN3*, were also upregulated, confirming the reliability of this transcriptome analysis. One of the novel, previously unpublished genes was *PAG1*, a transmembrane adaptor protein known to interfere with Src signalling (14,15). PAG1 attracted our attention because it had been reported to be overexpressed in clear cell renal cell carcinoma (ccRCC) which is frequently associated with loss of VHL function and constitutive HIF-2α overexpression (28). Furthermore, *PAG1* was among the high stringency genes with remote HIF-2 binding sites (7), suggesting that it may serve as prototype gene to study gene activation by remote HIF-2-dependent HREs.

We first confirmed hypoxic PAG1 induction in a panel of cancer cell lines derived from a broad range of different tissues. mRNA levels of both PAG1 and the well-known hypoxia-inducible CAIX were robustly upregulated by 3 to 11-fold in all cancer cell lines examined, except in VHL-deficient 786-0 ccRCC in which PAG1 levels were constitutively high (Figure 1A). Similar results were obtained on the protein level (Figure 1B). To analyze hypoxic induction of PAG1 *in vivo*, we quantified mRNA levels in various tissue samples derived from mice exposed to inspiratory hypoxia (8% O_2) for up to 108 hours. A profound time-dependent PAG1 upregulation could be observed in heart, lung, spleen and kidney with maximal induction factors ranging from 2 to 7-fold (Figure 1C).

Regarding the hypoxic PAG1 induction in mouse kidney, we next analyzed gene array data from human renal biopsy specimens collected from the European Renal cDNA Bank (21). Data were obtained from various nephropathies, including focal-segmental glomerulosclerosis (FSGS), rapidly progressive glomerulonephritis (RPGN), diabetic nephropathy (DN) as well as pre-transplant biopsies from living renal allograft donors as controls. Emerging evidence suggests that dysregulation of hypoxia-regulated transcriptional mechanisms contribute to the loss of renal function and the development of chronic kidney disease. A consistent and significant association of elevated PAG1 levels with advanced end-stage kidney disease was found for the glomerular as well as tubulointerstitial compartment (Supplementary Figure 1A). These gene array data were validated by RT-qPCR in an independent patient cohort, supporting the association of induced PAG1 levels and several advanced glomerulopathies (Supplementary Figure 1B). Hypoxically elevated PAG1 mRNA levels were also found in immortalized human cell lines derived from different renal compartments, including HK2 proximal tubular cells (Supplementary Figure 1C) and TK188 and TZ-1 renal fibroblasts (Supplementary Figure 1D). In summary, these data suggest that PAG1 is hypoxically induced under various physiological as well as pathological conditions affecting renal function.

PAG1-mediated basal regulation of Src signalling is not altered by hypoxic conditions

PAG1 has been reported to be involved in the regulation of Src family kinases (SFKs) (14,15). To address the potential functional consequences of PAG1 hypoxic regulation we used Hep3B cells, which contain a combination of relatively high basal and strong hypoxia-inducible PAG1 levels, and generated shPAG1 Hep3B cells. PAG1 knockdown efficiency was validated on the mRNA and protein levels (Supplementary Figure 2A). A proteome profiler phospho-kinase array screen did not reveal any PAG1-dependent difference in SFK activity, in shPAG1 compared with shCtrl Hep3B cells under normoxic or hypoxic conditions (Supplementary Figure 2B). Consistently, following PAG1 immunoprecipitation and detection with a pan-phosphotyrosine antibody, no change in specific PAG1 phosphorylation could be observed while total PAG1 protein levels were again increased (Supplementary Figure 2C).

PAG1 is a HIF target gene

The abundant and oxygen-independent PAG1 levels in 786-0 cells suggested PAG1 regulation by the VHL/HIF-2α axis. Indeed, both reconstitution of VHL as well as shRNA-mediated knockdown of HIF-2α not only reduced HIF-2α but also PAG1 mRNA and protein levels (Figure 2A).

To investigate a potential transcriptional selectivity for a specific HIFα isoform, PAG1 transcript levels were analysed in shHIF-1α and/or shHIF-2α HeLa and MCF-7 cell lines cultured under normoxic and hypoxic conditions. Hypoxic PAG1 mRNA induction was significantly attenuated by shHIF-2α in HeLa cells and entirely eliminated by shHIF-2α in MCF-7 cells (Figure 2B). HIF-1α shRNA had no significant effect on hypoxic PAG1 expression in MCF-7 cells and only a minor effect in HeLa cells. In both cell lines, combined HIF-1α/HIF-2α knockdown abolished hypoxic PAG1 induction (Figure 2B). Similar results were obtained for PAG1 protein levels (Figure 2C). Consistent with our previous findings (24,29), increased HIF-2α could be observed in the absence of HIF-1α, which further

induced hypoxic PAG1 protein levels (Figure 2C). These data demonstrate that at least in the cell lines analyzed PAG1 is predominantly regulated by HIF-2α.

Localization of a putative distal HRE -82 kb upstream of the *PAG1* TSS

In order to assess the molecular mechanism of HIF-mediated *PAG1* transactivation, we first analyzed a 1 kb promoter fragment upstream of the *PAG1* TSS, containing a single consensus HRE motif, in a reporter gene assay. However, in contrast to a similar construct driven by the established hypoxia-inducible PHD2 promoter (17), the PAG1 promoter was not induced by hypoxia (Figure 3A).

We next interrogated the genome-wide HBS ChIP-seq datasets (7) and identified an intergenic HIF-2α binding site 82 kb upstream of the *PAG1* TSS in both MCF-7 and 786-0 cells (Supplementary Figure 3). This site contains a consensus HRE motif, overlaps with DNase I hypersensitivity clusters that reflect open chromatin, and displays several epigenetic marks of active enhancers as indicated by the UCSC-integrated ENCODE data (Figure 3B). Furthermore, the remote site contains strong transcription co-factor occupancy, including p300 and RNApol2, as illustrated by the compressed transcription factor ChIP-seq ENCODE track in Figure 3B and expanded view in Supplementary Figure 3. During the course of our study this distant locus was reported as a HIF-1 binding site detected by ChIP-seq in HUVEC cells (9). ChIP-qPCR experiments for HIF-1α, HIF-2α and HIFβ in MCF-7 and 786-0 cells independently validated a robust binding of all three HIF subunits at this site (Figure 3C). Common binding of HIF-1 and HIF-2 is frequently observed at loci that are transcriptionally regulated by a single isoform, reflecting post-binding mechanisms of transcriptional selectivity (7). Consistent with the ENCODE data, ChIP-qPCR based analysis of histone marks revealed high levels of H3K27Ac and H3K4Me1, with low levels of H3K4Me3, in MCF-7 and 786-0 cells (Figures 3D and E). This combination of histone modifications is usually observed at active enhancers but not promoters. These results together with the lack of detection of any transcript in this region using MCF-7 based RNA-sequencing (30) exclude that this site is regulating a potential proximal unannotated transcript. Furthermore, substantial enrichment of this locus was observed upon transcriptional co-activator p300 precipitation (Figure 3D). p300 is a well-known essential transcriptional co-activator of HIFs (31).

Of note, the TSS of *FABP5* (fatty acid binding protein 5) is located 86 kb further upstream of the putative *PAG1* HRE (indicated in Figure 3F, left part), suggesting that *FABP5* might also be oxygen-regulated. However, in line with a previously reported study (32), RT-qPCR quantification of *FABP5* transcript levels did not reveal any hypoxic induction in the cell lines used above (Figure 3D, right part). Moreover, physical association experiments exclude an interaction of the intergenic -82 kb HRE with the *FABP5* promoter (J. Platt and D. Mole, unpublished observations). Collectively, these data indicate that the remote -82 kb HRE might be involved in the transcriptional regulation of *PAG1* but not the equally distant *FABP5* gene.

Hypoxic activation of reporter gene expression by the -82 kb *PAG1* HRE

To evaluate the functionality of the putative -82 kb HRE, heterologous reporter gene assays were performed using a 317 bp fragment encompassing the HBS and covering the entire high stringency HIF-2 binding site (7). This 317 bp fragment was cloned in both orientations upstream of a heterologous SV40 promoter (Figure 4A). pH3SVL, an SV40 promoter-driven luciferase reporter gene containing 3 concatamerized HREs derived from the -3.5 kb *transferrin* enhancer (33) was used as positive control. In contrast to pH3SVL, the 317 bp fragment did not significantly increase firefly luciferase activity in hypoxic HeLa cells (Figure 4A). However, a 2 kb fragment of the same -82 kb region significantly enhanced hypoxic reporter gene induction, regardless of its orientation (Figure 4A), confirming that the minimal HBS is not sufficient to constitute a functional HRE but rather includes additional *cis*-acting elements. Indeed, only the 2 kb fragment covers the entire ENCODE-derived H3K4Me1 and H3K27Ac tracks found in the UCSC Genome Browser (Supplementary Figure 3).

To assess HIFα isoform-specific hypoxic *trans*-activation of the 2 kb fragment encompassing the -82 kb *PAG1* HRE, single or double shHIFα-mediated knockdown cells were transfected with reporter gene constructs. Consistent with endogenous PAG1 protein regulation (see above), shHIF-1α/shHIF-2α double-knockdown HeLa and MCF-7 cells were not able to induce HRE-dependent luciferase activity (Figure 4B). *PAG1* -82 kb HRE-driven reporter gene activity was mainly impaired by shHIF-1α single-knockdown in HeLa cells and shHIF-2α single-knockdown in MCF-7 cells (Figure 4B), as observed for endogenous PAG1 expression shown above.

To explore the functional interaction of the *PAG1* promoter with the -82 kb HRE, luciferase reporter genes were constructed containing the 1 kb promoter fragment 5' of the luciferase gene and the 2 kb HRE fragment 3' to the luciferase gene to mimic its distal location on the circular plasmid (Figure 4C). An established PHD2 promoter-driven reporter gene was included as positive control (17). As observed before, the *PAG1* promoter alone conferred basal but not hypoxically induced

reporter gene expression. However, the -82 kb *PAG1* HRE significantly enhanced *PAG1* promoter activity under hypoxic conditions (Figure 4C). Mutation of the -82 kb HRE (5'-CGTG-3' to 5'-ATAA-3') completely abrogated hypoxic reporter gene induction (Figure 4C).

Finally, overexpression of hydroxylation-resistant HIFα isoforms in HeLa and MCF-7 cells confirmed HIF-mediated activation of reporter gene expression under the control of the -82 kb *PAG1* HRE (Figure 4D). The promoters derived from the genes encoding PAI-1 and CAIX served as controls for hypoxic induction preferentially driven by HIF-1 and HIF-2, respectively. Notably, overexpressed HIF-2α enhanced *PAG1* promoter-driven reporter gene expression substantially better than HIF-1α in both cell lines , and mutation of the -82 kb HRE again fully abrogated HIF responsiveness of the reporter gene (Figure 4D).

Destruction of the -82 kb *PAG1* HRE abrogates hypoxic PAG1 mRNA induction

Because in total 72 canonical 5'-RCGTG-3' HBS sequence motifs are located within the 82 kb region upstream of the *PAG1* TSS, it is well possible that additional functional HREs might also mediate hypoxic PAG1 induction. To analyze whether the -82 kb *PAG1* HRE is necessary for PAG1 regulation, we used the TALEN technique (34,35) to disrupt the -82 kb HRE in HeLa and MCF-7 cells. Two different pairs of TALEN targeting vectors (referred to as TPI and TPII) were constructed (Supplementary Figure 4A). Following co-transfection with TPI or TPII, HeLa and MCF-7 cells, either positive for EGFP fluorescence or puromycin resistance, were sub-cloned and the -82 kb HRE amplified by PCR. BsaAI resistant PCR products indicated destruction of the HBS within the -82 kb HRE due to *FokI* endonuclease cleavage followed by non-homologous end joining (NHEJ) DNA repair (Supplementary Figure 4B). Of 96 cell clones that were genotyped, 24 were found to be BsaAI resistant on either one or both alleles (Figure 5A, Supplementary Figure 4C). DNA sequence analysis revealed different NHEJ-mediated repair, demonstrating independent clones (Figure 5B).

PAG1 -82 kb HRE wild-type and mutant cell clones were cultured under normoxic or hypoxic conditions and PAG1 mRNA levels quantified. Whereas wild-type cells consistently displayed significant oxygen-dependent PAG1 expression levels, hypoxic inducibility of PAG1 mRNA was lost in all HeLa (Figure 5C, left part) and MCF-7 (Figure 5D, left part) cell clones analyzed, demonstrating that this distant HRE is solely responsible for hypoxic PAG1 regulation. In contrast, hypoxic CAIX mRNA induction remained unaffected (Figures 5C and D, right part), suggesting that the oxygen-sensing pathway is still intact in these newly generated sub-clones of the HeLa and MCF-7 cell lines. Consistently, EGFP positive or puromycin resistant clones which contained two wild-type or only one targeted allele still showed hypoxic PAG1 (and CAIX) mRNA induction (Supplementary Figures 4D and E). For HeLa clone 13 this apparent hypoxic induction was however not significant (Supplementary Figure 4D).

To further rule out any potential TALEN off-target effects, we performed reporter gene assays using the HIF-1-dependent *CA9* and HIF-2-dependent *PAI1* promoters to drive firefly luciferase gene expression in *PAG1* -82 kb HRE mutant HeLa and MCF-7 cells. Although some non-significant variability could be observed, HIF-1 and HIF-2 driven reporter gene activity was similar if not even more pronounced in these cells (Figures 5E and F), confirming that general changes in the HIF oxygen sensing pathway did not account for the TALEN-mediated loss of hypoxic PAG1 induction upon mutation of the -82 kb HRE.

The data obtained on PAG1 mRNA levels were confirmed by loss of hypoxic PAG1 protein induction upon -82 kb HRE mutation in HeLa (Figure 5G, Supplementary Figure 4F) and MCF-7 cells (Figure 5H, Supplementary Figure 4G). Quantification of protein levels again displayed significant hypoxically induced PAG1 expression levels in wild-type cells, which was absent in the mutant cells (Figure 5G and 5H, right panel). Unexpectedly, some of the targeted HeLa cell clones displayed increased basal PAG1 mRNA (Figure 5C, Supplementary Figure 4D) and protein (Figure 5G, Supplementary Figure 4F) levels, similar to the double shHIF-1α/shHIF-2α HeLa cells (Figure 2C). While we currently have no explanation for these clonal changes in PAG1 expression, they did not affect the conclusions regarding the function of the -82 kb HRE drawn from these experiments.

Whereas our TALEN data conclusively confirmed the functionality of the -82 kb HRE, comprehensive ENCODE analysis provided additional support for the functionality of the distal HRE in comparison to all other non-functional HREs in the upstream PAG1 region: only 3 of the 72 5'-RCGTG-3' motifs overlap with DNaseI hypersensitivity sites in more than 10 out of 125 cell lines analyzed (Version 3 in *hg19*), consistent with the earlier hypothesis that DNaseI hypersensitivity in normoxic cells represents an important predictor of HIF binding to its consensus recognition site. Indeed, it has been demonstrated previously that DNaseI hypersensitive 5'-RCGTG-3' motifs are 19 times more likely to bind HIF-1 and 22 times more likely to bind HIF-2 compared with DNaseI insensitive motifs (7). Importantly, none of the potential PAG1 upstream HRE sites, with exception of the promoter, contains a similar high transcription factor occupancy as the -82 kb HRE and none of the

72 5'-RCGTG-3' motifs coincides with a unique combination of histone mark H3K4Me1, strong transcription factor occupancy and robust DNaseI hypersensitivity (Supplemental Figure 3).

Chromatin interaction between the *PAG1* promoter and the -82 kb HRE enhancer is independent of HIF

To investigate the physical interaction between the -82 kb HRE with the *PAG1* promoter region, 3C assays were performed using the -82 kb enhancer as bait. Following cross-linking and EcoRI cleavage, re-ligation products were analyzed by unidirectional semi-quantitative PCR on agarose gels as indicated by the scheme shown on top of Figure 6. This method allowed the exclusion of incomplete EcoRI cleavage as a potential reason for fragment proximity. EcoRI digested and re-ligated BAC clones, covering the entire genomic region investigated, served as PCR efficiency controls, and the *EEF1G* locus was used to ensure equal crosslinking efficiency between the experiments.

PCR products were generally observed in MCF-7 (Figure 6A) and HeLa (Figure 6B) cells between the -83637 bp anchor primer and the +7631 bp as well as +1107 bp primers, both located in the first *PAG1* intron, suggesting that the -82 kb HRE directly interacts with the TSS of *PAG1*. Consistently, both intronic sites coincide with a DNaseI hypersensitivity signal in MCF-7, as deduced from publically available ENCODE data. These intronic interactions also largely corroborate with recent genome-wide RNApol2 binding studies showing that for the majority of hypoxia-inducible genes, RNApol2 is already bound at the promoter in normoxic cells and hypoxia does not substantially increase promoter-bound RNApol2, but rather leads to an increase in RNApol2 across the body of the gene (30), suggesting that HIF is involved in elongation of transcription rather than transcription initiation complex formation. In HeLa cells, an additional strong interaction could be observed between the distal enhancer and the most proximal promoter fragment (Figure 6B).

Interestingly, hypoxic exposure (0.2% O_2, 24 hours) of MCF-7 and HeLa cells did not significantly alter the distal chromatin interaction. We also detected considerable proximal chromatin interactions in both cell lines under normoxic as well as hypoxic conditions.

The lack of significant hypoxic changes in chromatin looping suggested that HIF is not involved in these interactions. Indeed, in shHIF-1α/2α MCF-7 cells (see Figure 2C) a similar, even more pronounced general pattern of chromatin looping could be observed (Figure 6C), providing further evidence that induction of HIF is not required for chromatin interactions with the -82 kb HRE.

Finally, we repeated the 3C experiments in MCF-7 and HeLa cells following TALEN-mediated destruction of the HBS within the -82 kb *PAG1* HRE. Chromatin interactions with the mutant HBS-containing EcoRI fragment generally became weaker in MCF-7 (Figure 6D) and HeLa (Figure 6E) cells when compared to the maternal cell lines (Figures 6A-B). However, most of these interactions still appeared more robust than the interaction frequencies of the HBS region with the EcoRI fragments in between the *PAG1* promoter and HRE regions. Of note, proximal and distal looping was similarly lowered, suggesting that at least some of the proximal loops required an intact HRE and did not merely occur by chance due to proximity.

DISCUSSION

In this paper, we report on the oxygen-dependent regulation of the *PAG1* gene which we newly identified by gene array analysis of hypoxic HeLa cells. PAG1 mRNA levels were robustly induced in various human cell lines and mouse tissues. This finding is quite remarkable because in tissues *in vivo* we generally do not observe similar strong expression of HIF target genes as in cell culture *in vitro* (19,36). However, we could not identify any effect on Src signalling following PAG1 induction by hypoxia. Upon phosphorylation by SFKs, PAG1 is known to associate with C-terminal Src kinase (Csk) through Tyr-317, proximal to membrane-associated SFKs (Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes and Yrk). Csk then phosphorylates the C-terminal negative regulatory tyrosine residue of SFKs, which suppresses their activation (37). In our hands, hypoxia neither affected PAG1 tyrosine phosphorylation nor the phosphorylation of any SFK member. This may be attributed to a certain degree of redundancy in PAG1 function because various PAG1-deficient mouse models did not reveal any obvious phenotype (38-40). It could also be that subtle PAG1-mediated changes in SFK function become apparent only at specific time points after the onset of receptor stimulation-mediated Src signalling. However, cell type-specific functional investigations of Src signalling were beyond the scope of the present work.

Because the *PAG1* promoter region did not confer hypoxic inducibility to a reporter gene, we looked for more distal HREs regulating *PAG1* gene expression. Pan-genomic ChIP-seq studies previously identified frequent distal HREs, including distant intronic, exonic and intergenic regions (7,11). We could identify a single -82 kb HRE that is essential for hypoxic *PAG1 cis*-activation, as shown by TALEN-mediated destruction of the HBS within this HRE. To our knowledge, this is the first time that gene editing has been used to functionally analyze an HRE in human cell lines. Using

homologous recombination in mouse ES cells, HREs regulating the *Vegf* and *Epo* genes have been studied previously. Deletion of the HRE located in the promoter of the *VEGF* gene unexpectedly resulted in adult-onset progressive motor neuron degeneration reminiscent of amyotrophic lateral sclerosis, probably due to neuronal hypovascularization (41). Mutation of the 3'HRE regulating the *Epo* gene revealed that this HRE, in contrast to previous belief, mediates hypoxic *Epo* induction in the liver but not the kidney (42). We recently resolved this finding by suggesting the existence of a conserved -9.2 kb HRE within the distal 5' kidney-inducible element (13).

In contrast to the frequent distal location of HREs suggested by HIFα ChIP-seq, only very few of these sites have been functionally studied so far. For example, upstream HREs -35 kb and -57 kb from the TSS, respectively, mediate the hypoxic induction of the *SLC2A3* (9) and *IGFBP3* (12) genes. The only gene comprehensively analyzed containing an HRE more distant to the TSS than the -82 kb *PAG1* HRE gene reported herein is the *CCND1* gene (encoding cyclin D1) whose HRE is located -220 kb upstream of the TSS (8). Based on genome-wide association studies to screen for population-based cancer susceptibility loci, this intergenic remote HRE has been identified as a susceptibility locus for renal cell carcinoma, regulating *CCND1* expression via HIF-2 specifically in VHL-defective renal cancer cells. The protective haplotype impairs HIF-2 binding and thus links oxygen-sensing with cell cycle control (8). This newly identified polymorphism did not include the consensus HBS, implying that additional elements within this HRE are required for proper *cis*-regulation of hypoxic gene expression.

Although a very recent study (30) reported that HIF-binding sites are accessible in normoxia, prior to HIF stabilization the existence of remote HREs raises several additional questions regarding their physical interaction with the promoter region: i.) what is the dynamics of this interaction and how does it vary between different cell types? ii.) Is it affected by different oxygenation, i.e. differences in HIF protein levels? iii) How precisely is the HRE locus kept in an open chromatin conformation, especially when it is not residing within a methylation-free CpG island? Using 3C assays in MCF-7 and HeLa cells, we found constitutive oxygen and HIFα independent chromatin interaction between the *PAG1* -82 kb HRE and promoter regions. Such a pre-formed chromatin loop, independent of conditional cell signalling, is in line with a previous report demonstrating that TNF-α-responsive enhancers are in contact with their promoters before signalling (43). In fact, in this genome-wide study pre-existing chromatin looping was suggested to be a strong predictor of gene induction which contributes to cell-type specific regulation of conditional gene expression.

Obviously, there is a need for additional *trans*-acting factors, binding at or near the consensus HBS, to confer constitutive oxygen-independent chromatin looping. TALEN-mediated destruction of the HBS apparently only partially abolished chromatin interaction with the promoter region, suggesting that *trans*-acting factors interacting with both the HBS itself as well as neighbouring sites are involved. We and others previously demonstrated that the canonical HBS can also be bound by ATF-1, CREB-1 and USF transcription factors (33,44-46). These factors may be involved in preventing CpG methylation of unoccupied HBSs outside of CpG islands under normoxic conditions, which is known to block the interaction with HIF (47,48). Alternatively, they may be involved in epigenetic chromatin modification as well as in chromatin looping. While we did not analyze additional transcription factors that may bind at or near the HBS in the -82 kb *PAG1* HRE, several constitutive local chromatin interactions between the HBS and adjacent regions were detected in the 3C assays. Although the probability to detect such interactions by chance substantially increases with proximity, we consistently observed these local loops in all cell lines containing a functional HRE consensus motif. Notably, genome-wide studies identified many different transcription factors binding in the region surrounding the -82 kb *PAG1* HBS (Supplementary Figure 3), suggesting the existence of a larger cluster of transcription factors binding within the HRE, potentially involved in i) increased recruitment of HIF-2α over HIF-1α to this locus; ii) keeping the locus in an open chromatin conformation even in the (normoxic) absence of HIF; and iii) enhancing the stability of the long-range enhancer-promoter interaction.

Collectively, our data provide further evidence that a fully functional HRE is defined by a core HBS motif interacting with HIFs as well as with additional proximal DNA binding motifs interacting with other *trans*-acting factors involved in the (co-)recruitment of transcriptional co-activators (*trans*-activation), local chromatin activity (epigenetic modification) and long-range DNA-DNA interactions (chromatin looping). Considering the striking instantaneous stabilization of HIFα protein upon hypoxic stimulation (49), it is likely of major physiological relevance that HREs remain in an open conformation and pre-contact their respective promoters in order to immediately initiate essential adaptation pathways to survive oxygen restriction.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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FIGURE LEGENDS

Figure 1. Oxygen-regulated PAG1 expression *in vitro* and *in vivo*. (**A**) The indicated cancer cell lines were exposed to 20% or 0.2% O_2 for 24 hours and mRNA levels of PAG1 (top panel) and CAIX (bottom panel) were determined by RT-qPCR. Results of at least 3 independent experiments are shown relative to β-actin control mRNA levels. Error bars correspond to the SEM and statistical analyses were performed with an unpaired Student's t-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). (**B**) Cells were treated as in (**A**) and protein levels of HIF-2α, HIF-1α, PAG1 and β-actin were determined by immunoblotting. (**C**) Groups of 3 C57Bl/6 mice were exposed to inspiratory hypoxia (8% O_2) for 12 to 108 hours. PAG1 mRNA levels in the organs indicated were determined by RT-qPCR and normalised to ribosomal protein S12 mRNA levels. Results are displayed as fold hypoxic induction, error bars correspond to the SEM.

Figure 2. HIF-dependent PAG1 expression. (**A**, left panel) PAG1 mRNA levels in VHL-deficient 786-0, VHL-reconstituted 786-VHL and shHIF-2α 786-0 ccRCC cells. HIF-2α and CAIX mRNA levels were included as controls. All mRNA expression levels were determined by RT-qPCR and displayed relative to the β-actin mRNA levels (n = 3), error bars correspond to the SEM. Statistical analyses were performed with one-way ANOVA and Dunnet's correction for multiple comparisons (*, $P<0.05$; **, $P<0.01$). (**A**, right panel) Protein levels of HIF-2α, PAG1 and β-actin were determined by immunoblotting of 786-0 cells. (**B**) HeLa and MCF-7 cells were stably transfected with shCtrl, shHIF-1α and/or shHIF-2α, and exposed for 24 hours to 20% O_2 or 0.2% O_2. PAG1, CAIX, HIF-1α and HIF-2α mRNA levels were quantified by RT-qPCR and shown relative to β-actin mRNA levels (n = 3), error bars correspond to the SEM. Statistical analyses were performed with one-way ANOVA and Dunnet's correction for multiple comparisons (*, $P<0.05$; **, $P<0.01$). (**C**) HeLa and MCF-7 cells were treated as in (**B**) and HIF-2α, HIF-1α, PAG1 and β-actin proteins detected by immunoblotting.

Figure 3. Identification of a HRE 82 kb upstream of the *PAG1* TSS. (**A**) A *PAG1* promoter-driven firefly luciferase reporter gene was transiently co-transfected into HeLa cells followed by exposure to 20% O_2 or 0.2% O_2 for 24 hours. An SV40 promoter and a *PHD2* promoter-driven luciferase vector served as negative and positive controls, respectively. Results are displayed as ratios of firefly to *Renilla* luciferase activities in relative light units (R.L.U.) from 3 independent experiments performed in triplicates, error bars correspond to the SEM. Statistical analyses were performed with unpaired Student's t-tests (*, $P<0.05$; **, $P<0.01$). (**B**) UCSC Genome Browser output *(hg18)* of the *PAG1* genomic region, illustrating the location of the putative -82 kb HRE. In addition, the ENCODE integrated regulation track containing H3K4Me1/3 marks, H3K27Ac marks, DNaseI hypersensitivity clusters and transcription factor ChIP-seq data are displayed. Colors in the histone mark tracks represent conventional ENCODE codes used to illustrate different cell lines. (**C**-**E**) ChIP-qPCR of normoxic 786-0 cells and hypoxic (0.5% O_2, 16 hours) MCF-7 cells using antibodies directed against HIF-1α, HIF-2α, HIFβ, p300 and histone modifications H3K27Ac, H3K4Me1 and H3K4Me3. The HRE of EGLN3/PHD3, located in the first intron, served as positive control. Statistical analyses were performed with one-way ANOVA and Dunnet's correction for multiple comparisons (*, $P<0.05$; **, $P<0.01$). (**F**, left panel) Scheme depicting the location of the putative -82 kb HRE between the TSSs of *PAG1* and *FABP5*. (**F**, right panel) The indicated cell lines were exposed to 20% O_2 or 0.2% O_2 for 24 hours and FABP5 mRNA levels were quantified by RT-qPCR and shown relative to β-actin mRNA levels (n = 3).

Figure 4. Hypoxic *cis*-activation of reporter genes by the -82 kb *PAG1* HRE. (**A**) A 317 bp or a 2 kb fragment, both including the -82 kb *PAG1* HRE, were used to enhance expression of a SV40 promoter-driven firefly luciferase reporter gene vector (pGL3p). pH3SVL, containing 3 HREs with tandem HBSs derived from the *transferrin* gene, was used as a positive control. Following transient co-transfection, HeLa cells were exposed to 20% O_2 or 0.2% O_2 for 24 hours. Results are displayed as ratios of firefly to SV40 promoter-driven *Renilla* luciferase activities in relative light units (R.L.U.) from 3 independent experiments performed in triplicates, error bars correspond to the SEM. Statistical analyses were performed with unpaired Student's t-tests (**, $P<0.01$; ***, $P<0.001$). (**B**) HeLa and MCF-7 cells, stably transfected with shCtrl, shHIF-1α and/or shHIF-2α, were additionally transiently transfected with the indicated reporter genes. Statistical analyses were performed with one-way ANOVA and Dunnet's correction for multiple comparisons (*, $P<0.05$; **, $P<0.01$). (**C**) The 2 kb HRE fragment, containing the wild-type (HBSw) or a mutant HBS (HREm), was used to enhance expression of a *PAG1* promoter-driven firefly luciferase reporter gene (pPAG1p). A *PHD2* promoter-driven luciferase vector served as positive control. Statistical analyses were performed with unpaired

Student's t-test (***, *P*<0.001). (**D**) HeLa and MCF-7 cells were transiently co-transfected with the indicated reporter gene vectors together with empty, HIF-1α and HIF-2α overexpression vectors. The *CA9* and *PAI1* promoter-driven luciferase vectors served as HIF-1α and HIF-2α, respectively, isoform-specific controls. Statistical analyses were performed with one-way ANOVA and Dunnet's correction for multiple comparisons (*, *P*<0.05; **, *P*<0.01). (**B**-**D**) Exposure to hypoxia and determination of reporter gene activity was performed as in (**A**).

Figure 5. TALEN-mediated destruction of the -82 kb *PAG1* HRE. (**A**) PCR analysis of representative HeLa and MCF-7 clones following co-transfection of two independent TALEN pairs (TPI and TPII) targeting the HBS within the -82 kb *PAG1* HRE. A 317 bp fragment encompassing the putative HBS was amplified by PCR and restriction digested with (lower panel) or without BsaAI (upper panel). (**B**) DNA sequence analysis of the cell clones shown in (**A**). Dashes indicate deleted bases. Wild-type (wt) and targeted HeLa (**C**) and MCF-7 (**D**) clones were exposed to 20% O_2 or 0.2% O_2 for 24 hours, followed by quantification of PAG1 and CAIX mRNA levels. Results of 3 independent experiments are shown relative to the β-actin control mRNA levels. Statistical analyses were performed with unpaired Student's t-tests (*, *P*<0.05; **, *P*<0.01). Targeted HeLa (**E**) and MCF-7 (**F**) clones were transiently transfected with the indicated reporter gene vectors. Exposure to hypoxia and determination of reporter gene activity was performed as described for Figure 4A. Proteins from HeLa (**G**) and MCF-7 (**H**) cultures treated as above were analysed for HIF-2α, HIF-1α, PAG1 and β-actin levels by immunoblotting (left panels). A single representative immunoblot is shown. Following quantification of 3 independent immunoblots, PAG1 band intensities were shown relative to the intensities of the β-actin bands (right panels). Results are displayed as mean ± SD of n = 3. Statistical analyses were performed with unpaired Student's t-tests (**, *P*<0.01; ***, *P*<0.001).

Figure 6. 3C assays of a 95 kb region of the *PAG1* gene comprising the first intron to 4.9 kb upstream of the -82 kb HRE, as schematically indicated. The grey bar represents the location of the anchor fragment containing the -82151 bp HRE, triangles correspond to primer locations adjacent to the EcoRI sites. MCF-7 and HeLa wild-type (**A** and **B**, respectively), shHIF-1α/2α MCF-7 (**C**), and MCF-7 and HeLa HBS-destructed (**D** and **E**, respectively) cells were exposed to normoxic (left panel) or hypoxic (right panel) conditions for 24 hours. Relative interaction frequencies were obtained in triplicate from three independent experiments, error bars correspond to the SEM.

SUPPLEMENTARY DATA

Supplementary Table 1. PCR primers used for the amplification of the -82 kb *PAG1* HRE region.

Supplementary Table 2. ChIP-qPCR primers.

Supplementary Table 3. 3C primers (position relative to *PAG1* TSS).

Supplementary Table 4. Top 100 genes induced in HeLa cells following exposure to hypoxia (0.2% O_2) for 16 hours. Microarray data are shown as fold change (mean values of biological duplicates).

Supplementary Figure 1. PAG1 is induced in CKD patients and in hypoxic human kidney cell lines. (**A**) Gene array expression data were obtained from microdissected glomeruli and tubulointerstitial compartments from patients with glomerulopathies. Values are fold changes relative to living donors for 3 different probesets. (**B**) PAG1 mRNA levels were validated by RT-qPCR in an independent cohort of microdissected samples. PAG1 mRNA levels are shown relative to 18S rRNA. The number of patients is indicated between brackets. LD, living donors; DN, diabetic nephropathy; RPGN, rapidly progressive glomerulonephritis; FSGS, focal-segmental glomerulosclerosis. PAG1 mRNA (**C** to **E**) and protein levels (**C**) were determined in HK2 (**C**) TK188 (**D**) and TZ1 (**E**) cells following exposure to 0.2% O_2 for 24 hours. PAG1 mRNA levels are shown relative to ribosomal protein L28 mRNA levels, error bars correspond to the SEM.

Supplementary Figure 2. PAG1 phosphorylation is not affected by hypoxia. (**A**) PAG1 protein (left panel) and mRNA (right panel) levels as determined by immunoblotting and RT-qPCR following stable transfection of Hep3B cells with shPAG1 or shCtrl. PAG1 mRNA levels are shown relative to ribosomal protein L28 mRNA levels, error bars correspond to the SEM. (**B**) Human Phospho-Kinase array incubation with lysates of shCtrl or shPAG1 Hep3B cells following exposure to 20% O_2 or 0.2% O_2 for 24 hours. Dark dots on the upper and lower corner of the membranes represent undisclosed positive controls. The rectangles indicate the following SFKs: Src, Lyn, Fyn and Yes (duplicates; clockwise from upper left). (**C**) PAG1 tyrosine phosphorylation in Hep3B cells exposed to 20% O_2 or 0.2% O_2 for 24 hours. Anti-PAG1 or IgG control antibodies were used for Immunoprecipitation, followed by immunoblotting and detection with a pan-phosphotyrosine antibody.

Supplementary Figure 3. ChIP-sequencing reads covering the -82 kb *PAG1* HRE. UCSC Genome Browser output *(hg19)* indicating the HIF ChIP-sequencing reads for HIF-1α, HIF-2α and HIFβ in 786-0 and MCF-7 cells. Below a closer view is shown of the 82 kb *PAG1* HRE surrounding region with ENCODE-integrated DNase hypersensitivity clusters, histone marks and transcription factor occupancy.

Supplementary Figure 4. Destruction of the -82 kb *PAG1* HRE abolishes hypoxic PAG1 mRNA induction but not CAIX mRNA or HIFα protein induction. (**A**) Overview of the two pairs of TALEN vectors (TPI and TPII) used to target the -82 kb *PAG1* HRE. The core HBS and FokI cleavage sites are indicated. (**B**) Strategy to detect TALEN-mediated destruction of the HBS within the -82 kb *PAG1* HRE following NHEJ DNA repair. (**C**) Genotyping of representative targeted HeLa (left panel) and MCF-7 (right panel) clones by PCR amplification of the -82 kb *PAG1* HRE region followed by BsaAI restriction digestion and agarose gel electrophoresis. Following exposure of HeLa (**D**, **F**) and MCF-7 (**C**, **G**) clones to 20% O_2 or 0.2% O_2 for 24 hours, PAG1 and CAIX mRNA levels (**D**, **E**) as well as HIF-2α, HIF-1α, PAG1 and β-actin protein levels (**F**, **G**) were determined by RT-qPCR and immunoblotting, respectively. Transcript levels are shown relative to β-actin mRNA levels (n = 3), error bars correspond to the SEM. Statistical analyses were performed with unpaired Student's t-tests (*, P<0.05; **, P<0.01; ***, P<0.001).

Supplementary Table 1

Primer	Sequence (5’-3’)
317 bp fwd	TGGAAGATCTTGCCAATAATAGCATGCTGG
317 bp rev	TGGAAGATCTTTACAATAATTAGAGGCTTT
Enh_2 kb fwd	TGGAAGATCTTTACCTCCCAGTTGTCTGAAC
Enh_2 kb rev	TGGAAGATCTCTTGTGCCAAATCAGGCTAT

Supplementary Table 2

ChIP-qPCR primer	Primer fwd Sequence (5’-3’)	Primer rev Sequence (5’-3’)
β-actin	ACCATGGATGATGATATCGCC	GCCTTGCACATGCCGG
EGLN3	AGTGTCCGTTCCCAGCTCAG	TAGGCACAGTAAACAGGCC
PAG1 enhancer	TGCCAATAATAGCATGCTGG	TACAATAATTAGAGGCTTT

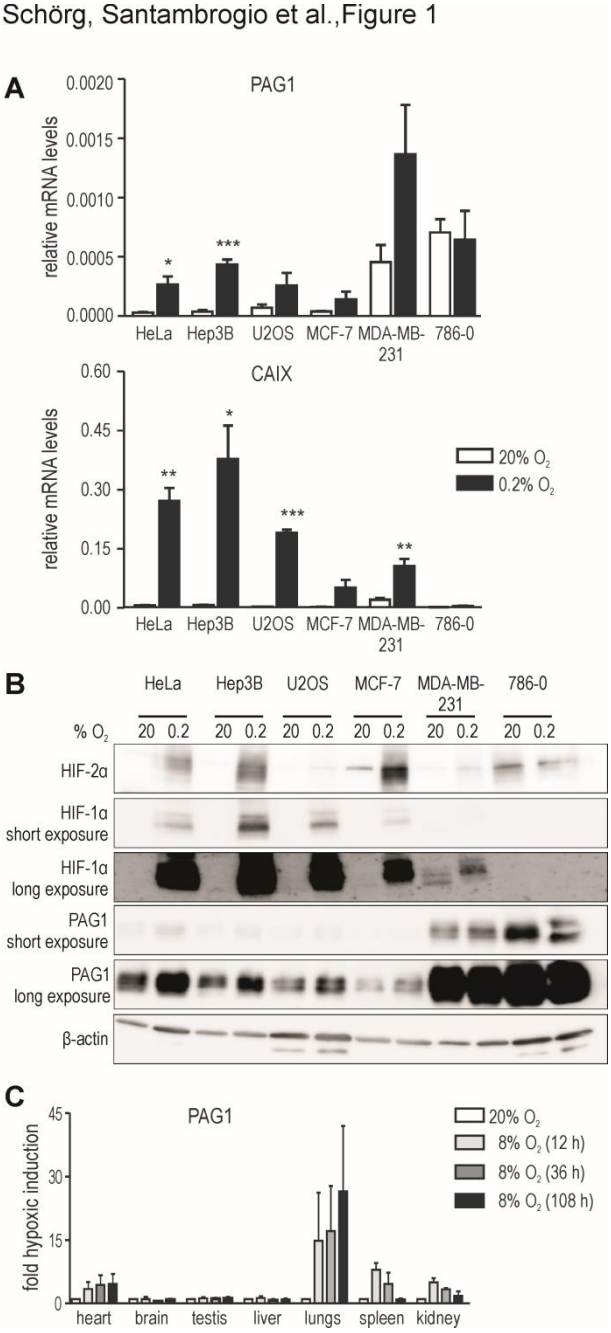
Supplementary Table 3

3C primer	Sequence (5'-3')
Anchor (-83637)	AAACACAGTAAATTCTCCAAAGCAG
7631	AACAACAATTTCAAATACACGGAAG
1107	TGAAATCCAAGTGTCATTATCTGAA
-665	ATCCTTGCTACTTAACGTGTGATCT
-2216	TCTAACTCTTCCATTACAGCCATTC
-2700	CAGCTACATCTTTTTCTCTGAGGTC
-5198	TTTTCTGAAAGAATCAGCCATTTAC
-17851	TACAAAATTGTCCTTCAGAAGACCT
-80655	TCACGTAAACCTTCAGTTTTCCTAC
-81310	CCATGTTAACCAAAGAGGTATCATC
-84203	TAATCTACCCCTTGTTTAGCATGTC
-86938	GCTTAATTTTAGATGAGAAGGCTCAA

Supplementary Table 4

Number	Gene	Fold change	Number	Gene	Fold change
1	CA9	74.76	51	SFXN3	3.1
2	PPFIA4	28.33	52	GOLGA8A	3.092
3	PFKFB4	18.62	53	KCTD11	3.053
4	NDRG1	18.38	54	HEY1	3.051
5	ANGPTL4	15.22	55	JMJD1A	3.022
6	SPAG4	11.89	56	MAFF	3.01
7	IGFBP3	8.351	57	HK2	2.968
8	EGLN3	7.464	58	B3GNT4	2.966
9	LOX	7.25	59	WDR54	2.957
10	ITGB4	7.16	60	DDR1	2.94
11	ERRFI1	6.346	**61**	**PAG1**	**2.932**
12	STC1	6.193	62	SFXN3	2.907
13	ADM	6.12	63	CXCR4	2.881
14	BNIP3L	5.845	64	FOS	2.854
15	SLC2A3	5.56	65	RNASE4	2.812
16	INSIG2	5.532	66	HILPDA	2.798
17	LOXL2	5.307	67	SH3PX3	2.787
18	SLC2A14	5.253	68	PPP1R3E	2.787
19	TMEM45A	5.201	69	ERRFI1	2.771
20	DLX4	4.92	70	GOLGA8A	2.767
21	ALDOC	4.896	71	RAB40C	2.76
22	AK3L1	4.768	72	AK3L1	2.708
23	CCNG2	4.673	73	PLOD2	2.706
24	CRABP2	4.502	74	DDIT4	2.674
25	MYO7B	4.271	75	GOLGA9P	2.674
26	PPP1R3B	4.172	76	SPTLC2L	2.649
27	ADFP	4.115	77	TPBG	2.648
28	MUC1	4.053	78	ANO7	2.647
29	ANKRD37	4.047	79	TMPRSS3	2.583
30	GPR146	3.992	80	TIMM13	2.573
31	KIAA1199	3.86	81	ARID5A	2.561
32	S1PR4	3.792	82	HK1	2.556
33	BHLHB2	3.733	83	TTYH3	2.555
34	FAM13A1	3.577	84	FAM162A	2.523
35	IER3	3.5	85	ATP1B1	2.515
36	VLDLR	3.5	86	OSMR	2.506
37	ITGA5	3.462	87	GBE1	2.5
38	ARRDC3	3.456	88	FAM83A	2.483
39	PPP2R5B	3.423	89	FAM139A	2.476
40	PDK1	3.374	90	BTG1	2.464
41	ANKZF1	3.354	91	CNOT8	2.441
42	PDK1	3.354	92	SLC6A6	2.433
43	TMPRSS3	3.353	93	PGK1	2.408
44	MXI1	3.304	94	FAM57A	2.387
45	AK3L1	3.301	95	GPRC5A	2.357
46	BHLHB2	3.252	96	C8orf58	2.345
47	TMEM145	3.24	97	FOXN4	2.335
48	ANG	3.216	98	QSOX1	2.327
49	ZNF395	3.15	99	ITGB8	2.325
50	WFIKKN1	3.121	100	RRAGA	2.317

Schörg, Santambrogio et al.,Figure 1



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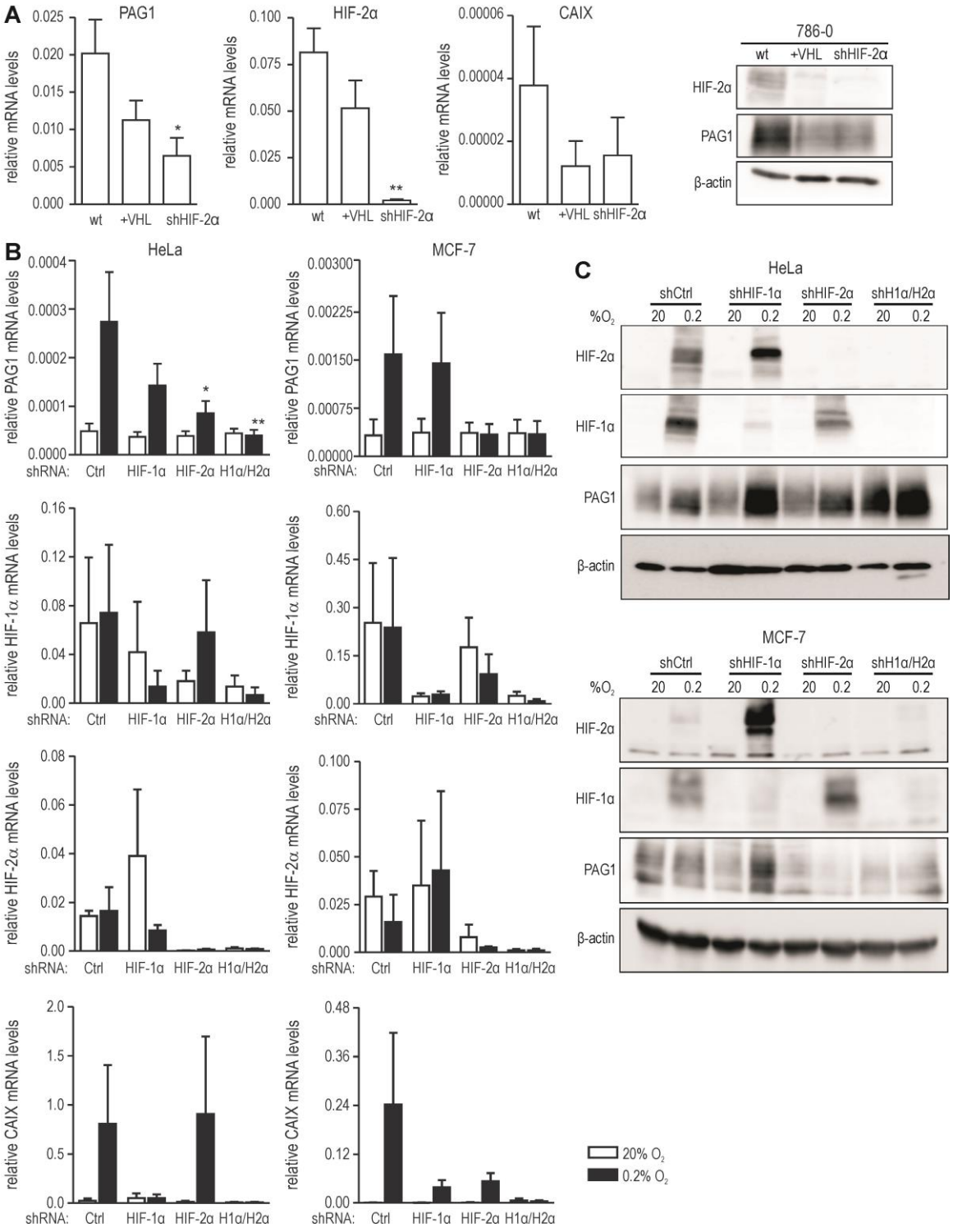

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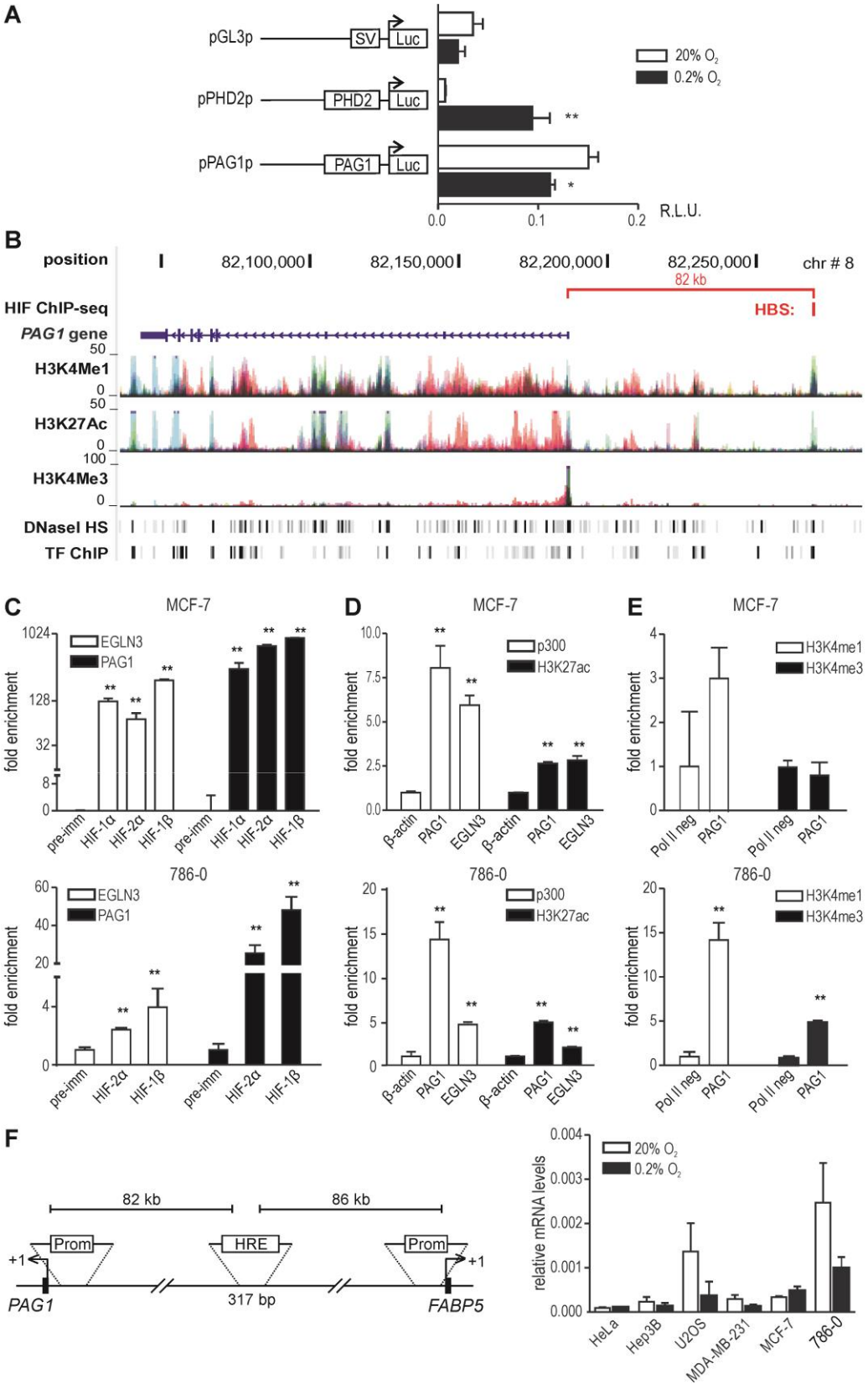

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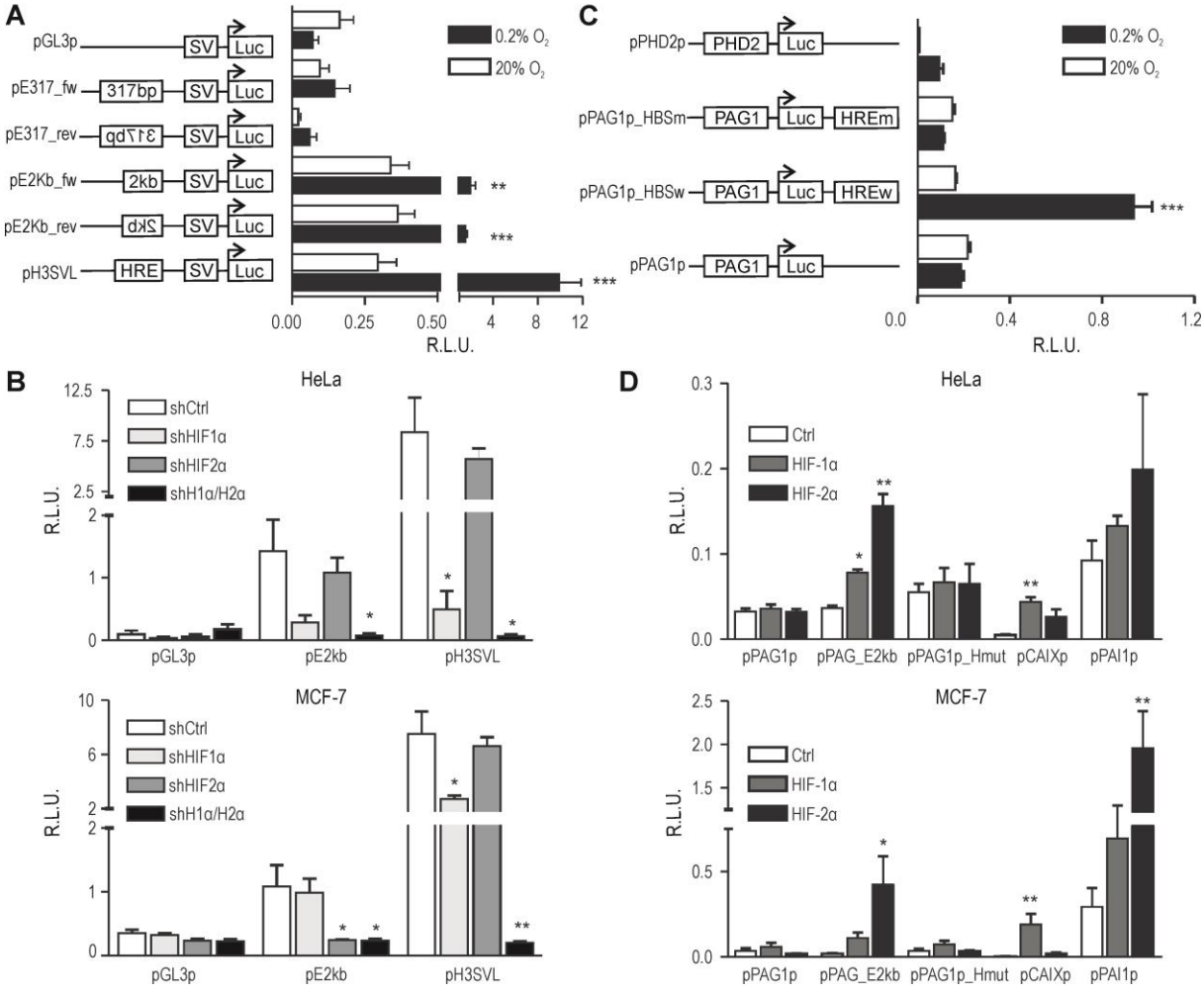

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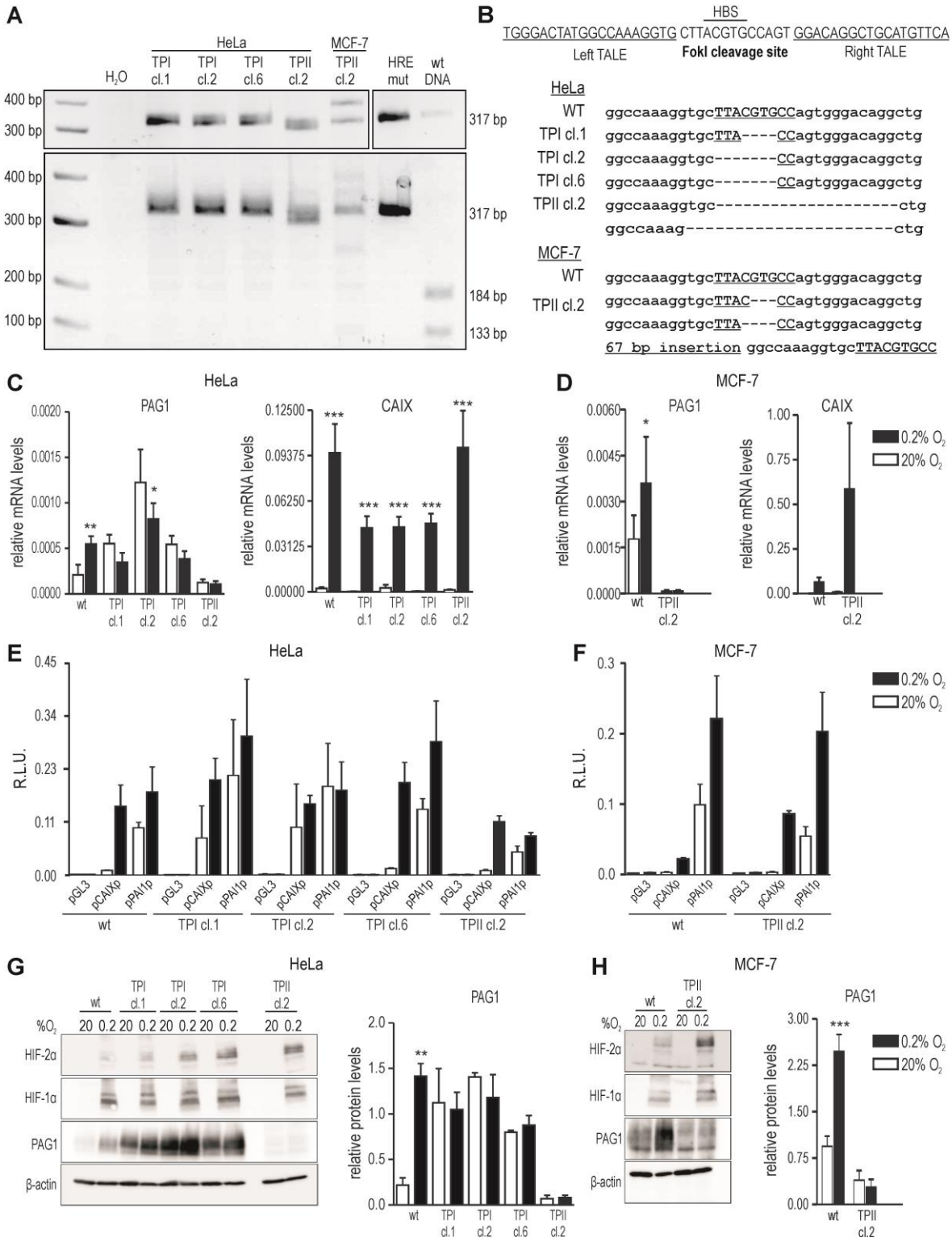

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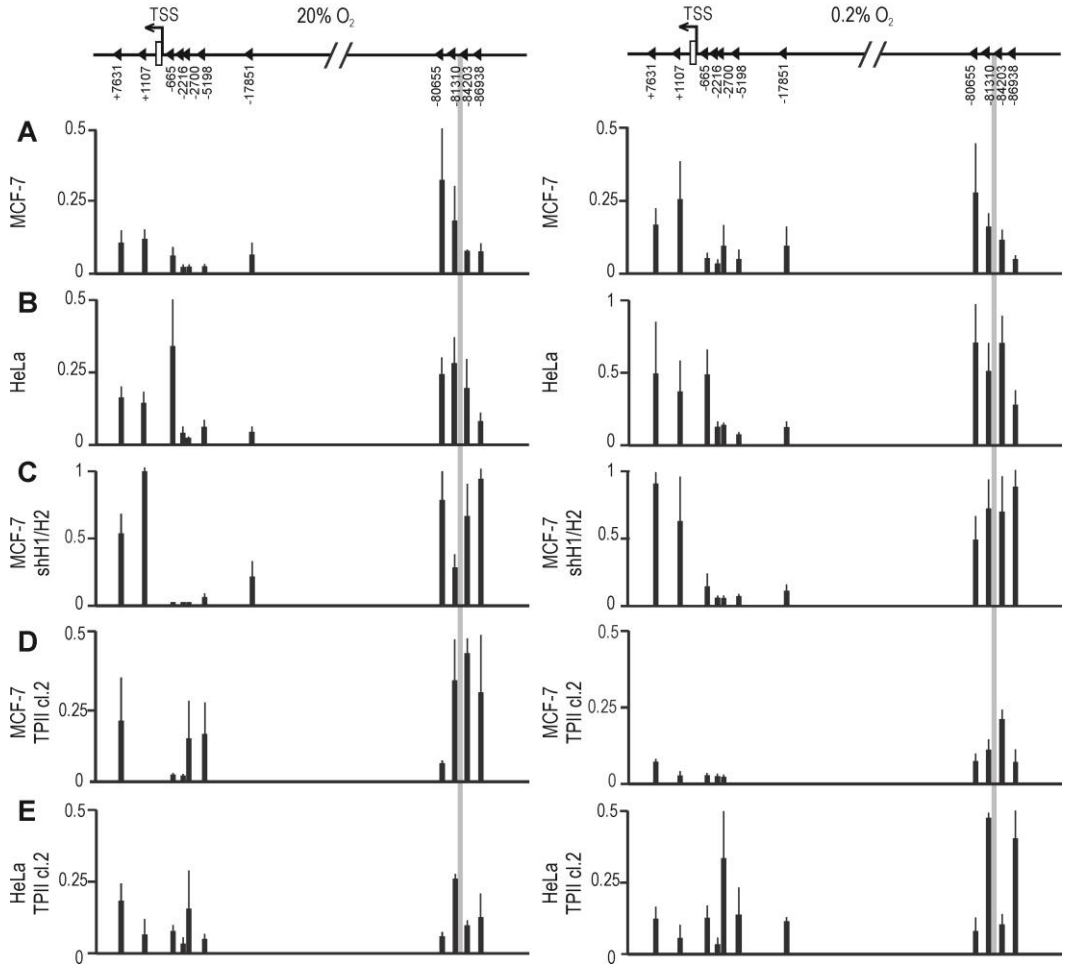
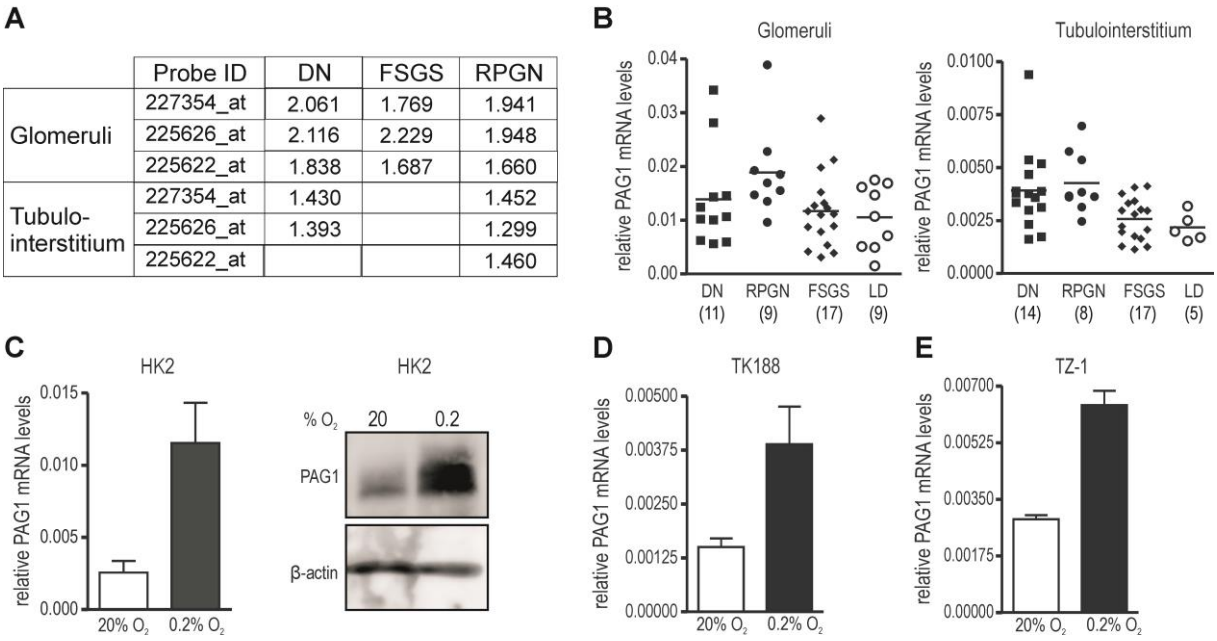


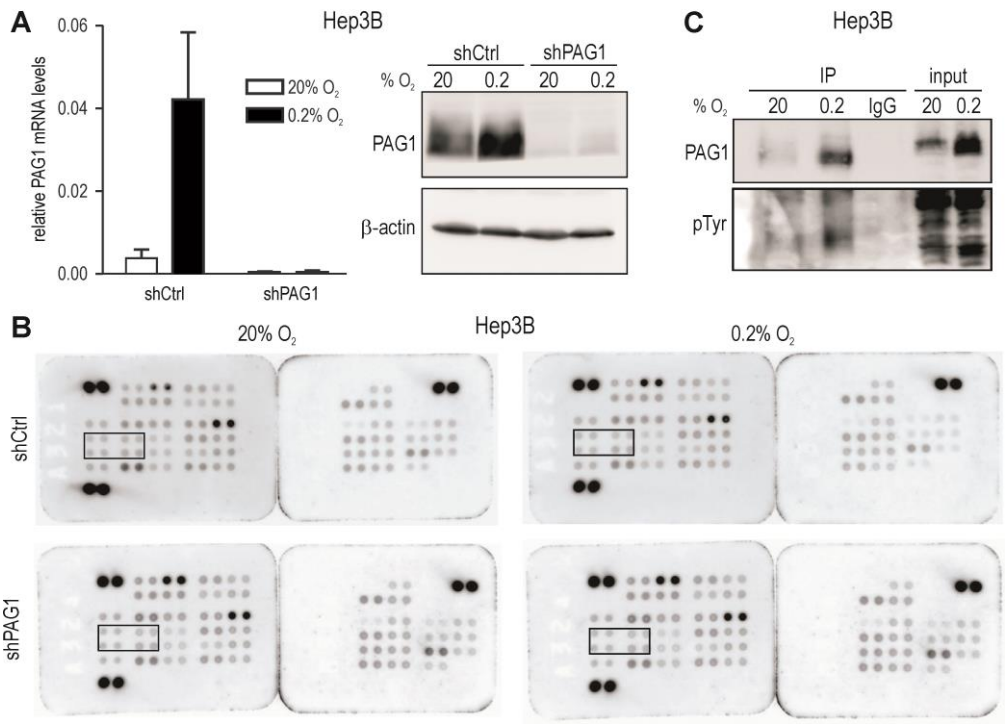
Schörg, Santambrogio et al., Supplementary Figure 1

A

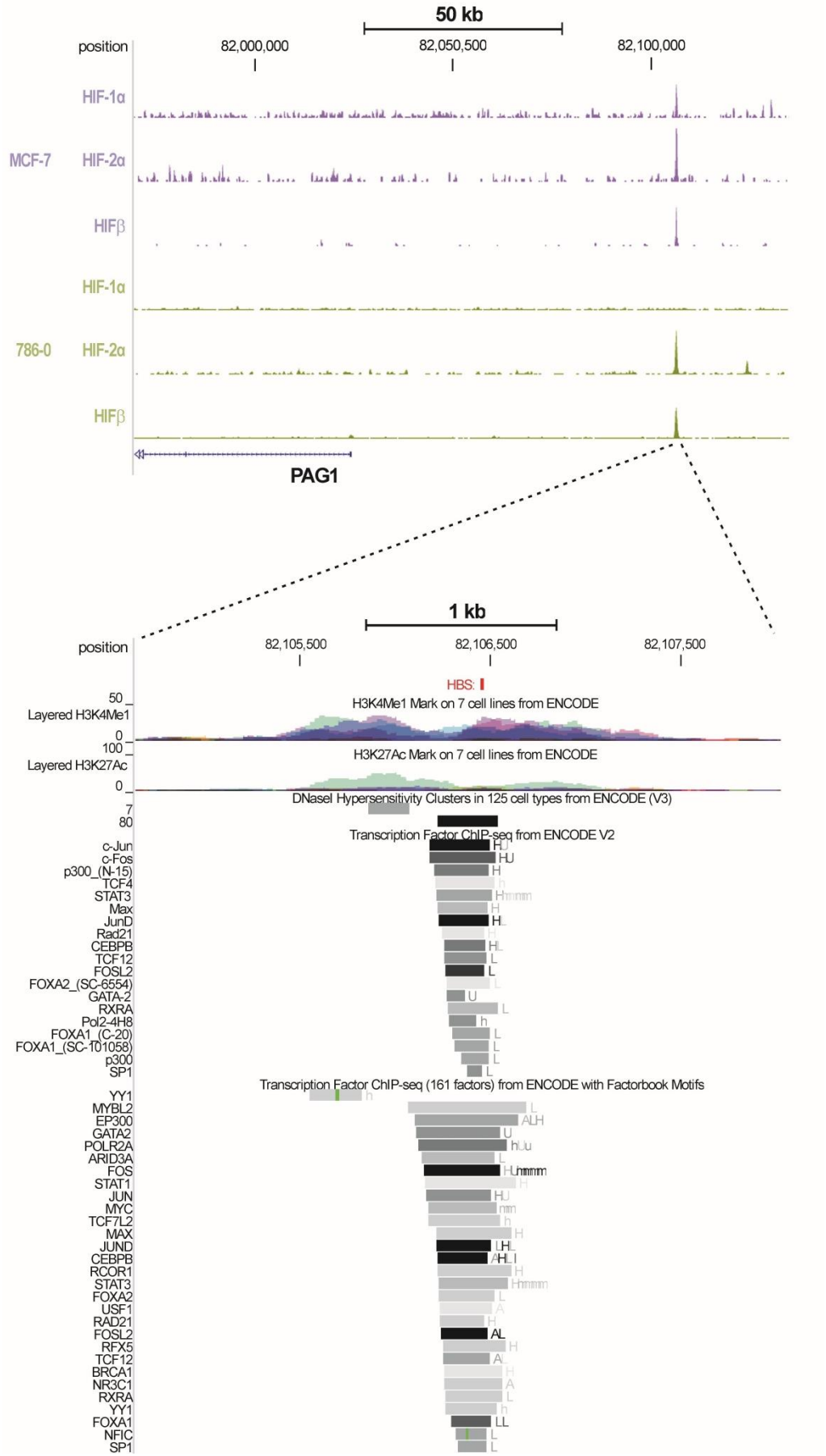
	Probe ID	DN	FSGS	RPGN
Glomeruli	227354_at	2.061	1.769	1.941
	225626_at	2.116	2.229	1.948
	225622_at	1.838	1.687	1.660
Tubulo-interstitium	227354_at	1.430		1.452
	225626_at	1.393		1.299
	225622_at			1.460



Schörg, Santambrogio et al., Supplementary Figure 2



Schörg, Santambrogio et al., Supplementary Figure 3



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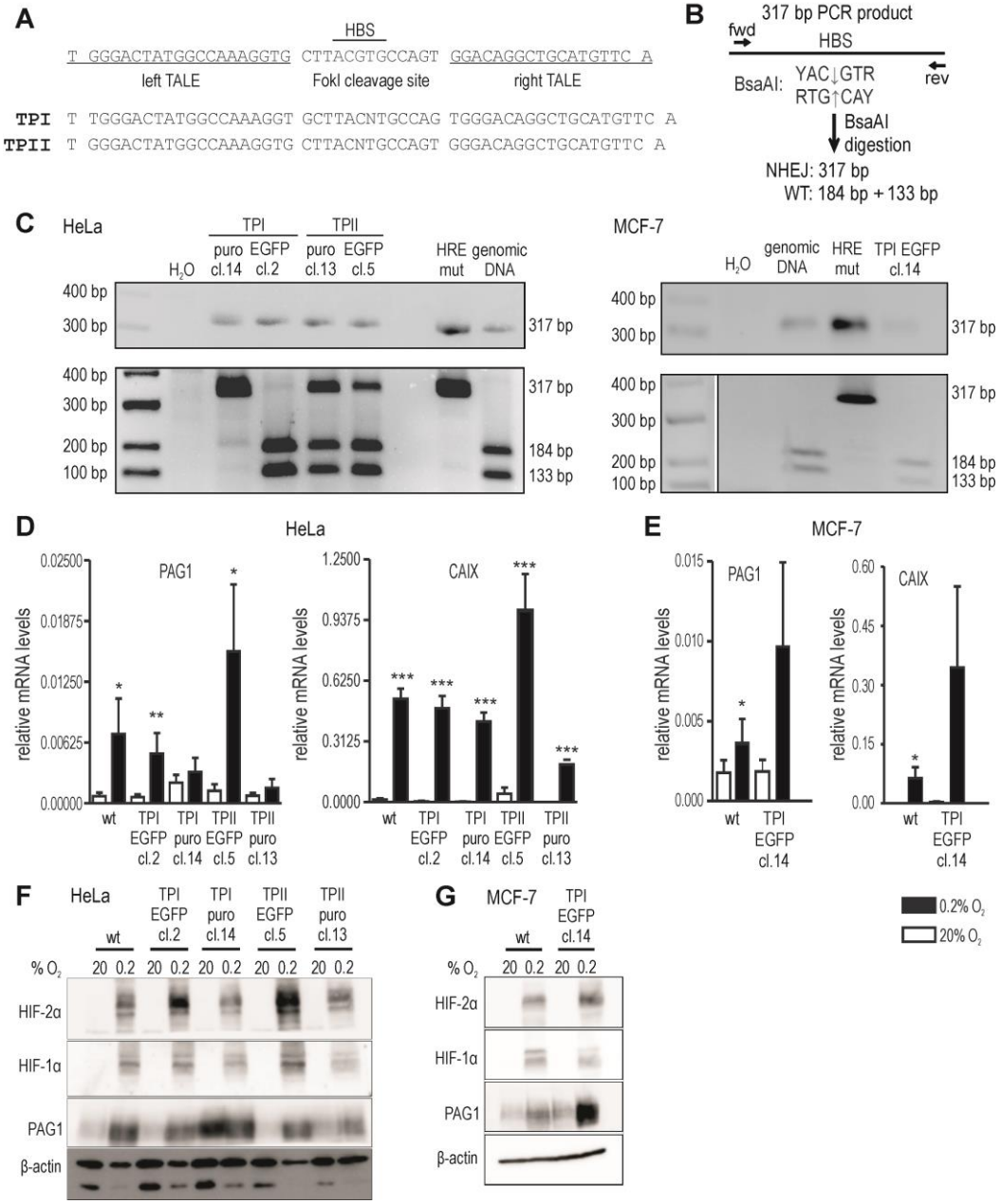

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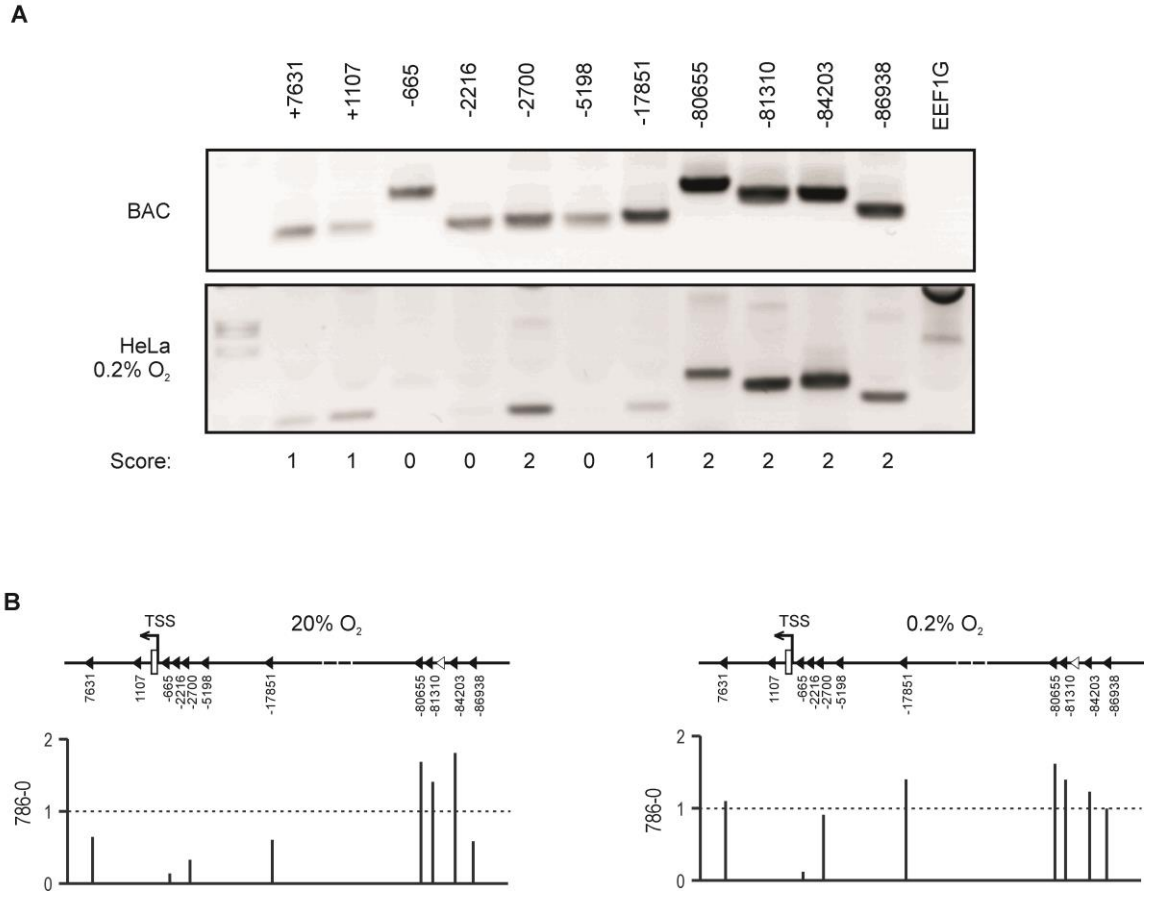

4. unpublished results

4.1. Cell line based screen for PAG1/Cbp expression in normoxia and hypoxic induction

Materials and methods:

HeLa, U2OS, MCF-7, Hep3B, A459, MDA-MB-468 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). THP1, HL-60 and Jurkat cells were cultured in Roswell Park Memorial Institute Medium (RPMI). All media were supplemented with 10% heat inactivated Fetal Calf Serum (FCS) and antibiotics penicillin 50 UI/ml and streptomycin 100 µg (Sigma). Hypoxia experiments were performed with the use of a gas controlled Invivo$_2$ 400 workstation (Ruskin) at 0.2% O_2. For mRNA expression total RNA was isolated from cells. Complementary DNA was generated by reverse transcription (RT) of 500 ng-2 µg of total RNA, using AffinityScript reverse transcriptase (Agilent). Transcript levels were determined by real-time quantitative PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Agilent). All RT-qPCR data are presented as ratios relative to ribosomal protein L28 mRNA values.

Protein analysis was performed as described in 3. , Material and Methods section.

Results

A screen for PAG1 expression levels in cancer cells from different origins was performed in normoxia. To have a variety of tissues present the cervical cancer cell line HeLa, the osteosarcoma cell line U2OS, the lung carcinoma line A549 and the hepatoma cell line Hep3B were investigated. Besides the estrogen receptor expressing low invasive breast cancer cell line MCF-7 and two high invasive, triple negative cell lines MDA-MB-468 and MDA-MB-231 were used to complete the panel. PAG1 is expressed at different levels in these cell lines (Fig.1A). The highest expression levels of PAG1 can be observed in MDA-MB-231 cells, followed by U2OS cells.

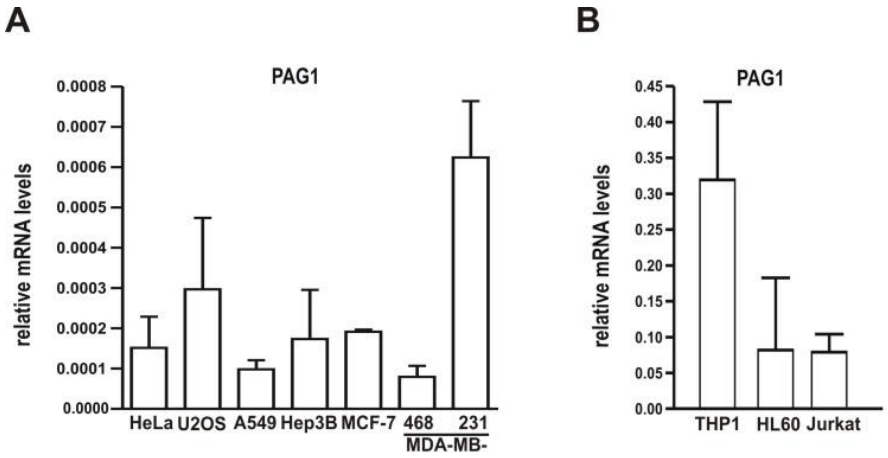

Fig. 1: PAG1 mRNA expression in a panel of cancer cell lines

(A) PAG1 mRNA in non-immune cancer cell lines reveals different expression levels. Total RNA was isolated from indicated cell lines and mRNA expression levels were determined by RT-qPCR. Gene expression levels were normalised to ribosomal L28 mRNA.

(B) PAG1 mRNA expression in immune cell derived cancer cell lines. Expression levels were analysed as in (A).

We also performed mRNA expression analysis in three immune cell cancer cell lines. Jurkat (derived from acute T-cell lymphoma) HL60 (a myeloid cell line) and THP1 (a monocytic cell line) were screened for PAG1 mRNA expression (Fig.1B). The highest expression could be observed in THP1 cell, whereas Jurkat and HL60 cells showed lower levels. Overall the basal expression in immune cells is about 2 orders of magnitude compared to the rest of the non-immune cell lines (Fig.1A). Furthermore we wanted to investigate the influence of hypoxia on the PAG1 expression in the immune system-derived cells. For this purpose we measured the mRNA expression levels of PAG1 in HL60 (Fig.2A) and Jurkat cells (Fig.2B) subjected to hypoxia.

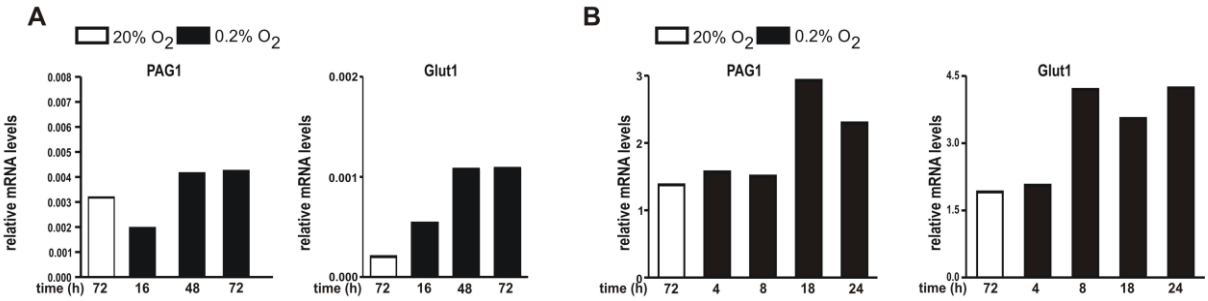

Fig. 2: PAG1 mRNA expression kinetics in immune-cell derived cell lines (n=2).

(A) HL60 cells were subjected to 0.2% or 20% O_2 for the indicated period of time. Total RNA was extracted and mRNA expression levels were determined by RT-qPCR. Gene expression levels were normalised to ribosomal L28 mRNA. PAG1 mRNA was measured and Glut1 mRNA served as hypoxic control.

(B) Jurkat cells were subjected to 0.2% or 20% O_2 for the indicated time points. Expression levels were confirmed as in (A).

Both measured genes showed an increase upon hypoxic exposure in both cell lines. In HL60 cells the PAG1 mRNA expression shows a 2 fold increase at 48 hours, where the level remains stable at 72 hours (Fig.2A). The Glut 1 expression is increased in hypoxia with a plateau at later time points (48 hours and 72 hours), confirming appropriate hypoxia. In the T-cell lymphoma line Jurkat PAG1 mRNA has a higher basal expression level, compared to HL60. The PAG1 mRNA shows a high peak after 18 hours hypoxia, followed by a reduction at 24 hours. Since most of the hypoxia regulated genes are not only up-regulated on the mRNA level, but also on the protein level, we investigated also protein expression in hypoxia in some of the cancer cell lines.

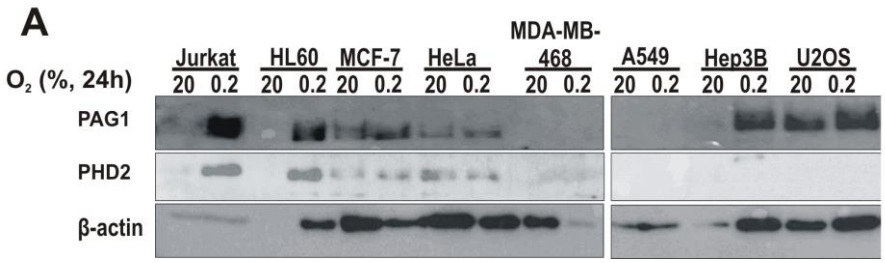

Fig. 3:PAG1 protein is up-regulated in hypoxia in some cancer cell lines

Comparison of PAG1 protein levels between cells subjected 20% O_2 or 0.2% O_2 for 24 hours.
Protein extracts were analysed by immunoblotting.

Fig. 3 shows the results of the protein analysis comparing PAG1 level between normoxic and hypoxic cells. The PHD2 protein induction in hypoxia was used as control for hypoxic exposure. Since not all

samples show an equal β-actin levels, which were used as loading control, these results have to be regarded as non-conclusive.

Discussion

The PAG1 mRNA is widely expressed, what we confirmed in our study. These findings are supported by a study of Oneyama and colleagues, were they analysed different cancer cells for PAG1 protein levels. They also found the highest levels in MDA-MB-231 and HEK293 cells. In contradiction to the presented results, in their study they could not show any detectable protein in MCF-7 cells, although here we could show that the PAG1 mRNA is present in these cells. In addition we show an up-regulation of PAG1 mRNA and protein levels in hypoxia compared to normoxia (see 3.). Furthermore we also observed high PAG1 basal expression levels in immune cell cancer cell lines, where we also show in kinetics that PAG1 mRNA is regulated by hypoxia. In Jurkat cells also the protein is up-regulated in hypoxic conditions.

4.2. Further characterisation of the hypoxia regulated *PAG1* enhancer element

Materials and Methods

Cell culture, hypoxia experiments, transfection and creation of the HeLa and MDA-MB-231 HIF knockdowns was performed as described previously (see 3.). Analysis of the CpG methylation state of the PAG1 HBS is described as followed. MCF-7, MDA-MB-231, HeLa and 786-O cells were subjected to 20% O_2 or 0.2% O_2 for 24 hours. Genomic DNA was isolated and 400 ng was subjected to BsaAI (New England Biolabs) digestion overnight at 37°C. 3 µl of digestion was used for subsequent Phusion-based PCR (Finnzyme) using the 317 bp primers as described before (see manuscript). To control the PCR reaction the same DNA was used in a PCR with L28 primers as listed below.

L28 fw	tttcccctcactctcattcg
L28 rev	ggatctccgcttaatgacca

mRNA, protein analysis and Dual-Luciferase assay were performed as described previously (see manuscript).

Results

HIFs are methylation sensitive transcription factors. Different methylation patterns in normoxia and hypoxia could explain the differential up-regulation levels in cancer cell lines from different tissue origin. To investigate this issue we developed a screen as depicted in Fig. 3A.

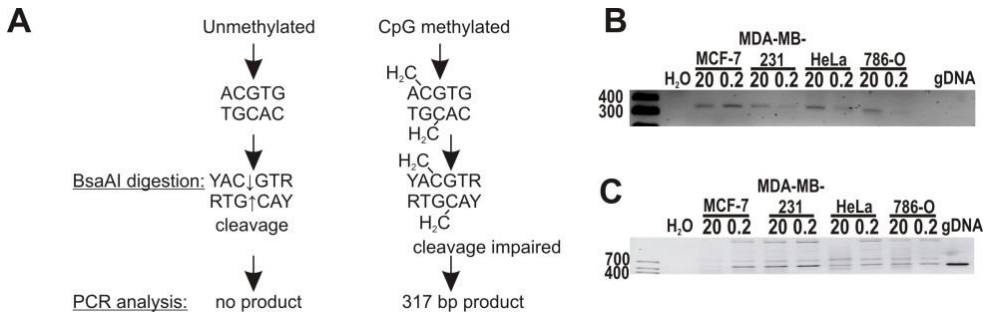

Fig. 3: Identification of potential CpG methylation at the HRE

(A) Flow chart indicating the experimental procedure. **(B)** PCR analysis of the pre-digested genomic DNA from cells, subjected to either 20% O_2 or 0.2% O_2 for 24 hours, resulting in 317bp product. **(C)** PCR analysis of the DNA used in (B) with L28 primers to control the PCR in (B), with an expected product of 578 bp. Results are exemplified pictures of two independent experiments.

In Fig. 3B the results for the PAG1 HRE are shown. Different intensities of the PCR band in between cell lines and hypoxic exposure can be observed. So this approach is able to discriminate between the methylation status of the HBS. Furthermore the analysis shows differences between cell lines and hypoxic exposure. The PCR amplifying a region in the L28 locus resulted in multiple bands due to the BsaAI pre-digestion of the genomic DNA. So another control should be taken into consideration.

HIF collaborate with a variety of cofactors to induce target gene expression, either via direct protein-protein interaction or binding to neighbouring DNA sequences. To investigate this topic we established a reporter gene based screen for the *PAG1* HRE including a set of factors already known to contribute

to HIF target gene regulation. The readout scheme is depicted in Fig.4A. The two endogenous *PAG1* promoter containing constructs including the enhancer element with wild type HRE or mutated HRE are overexpressed in HeLa cells together with the indicated transcription factors in Fig.4B.

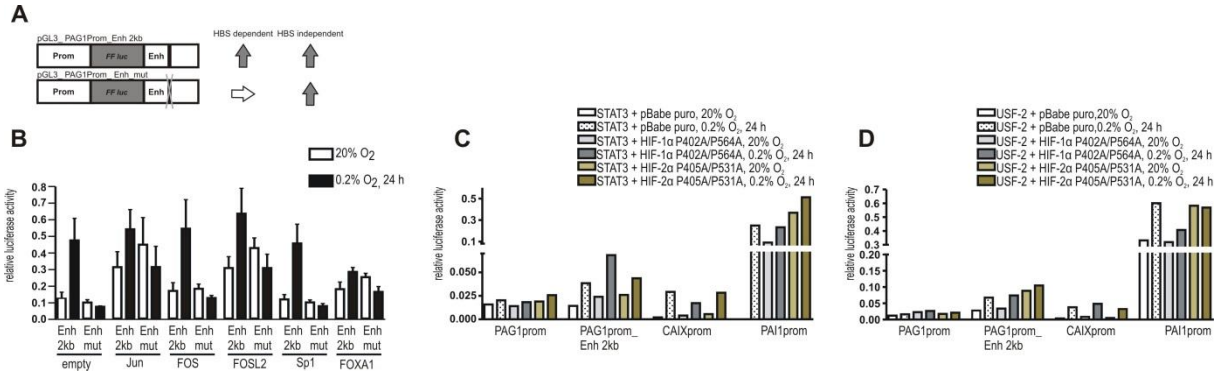

Fig. 4: Identification of potential cooperative transcription factors involved in the hypoxic response

(A) Schematic overview of the *PAG1* enhancer constructs used for the luciferase reporter gene assay based approach. Expected readouts for HBS-dependent and independent reporter gene activation are indicated by a cross table. **(B)** Luciferase based screening approach with the indicated constructs in A and the described transcription factors were transiently transfected into HeLa cells. 16 hours post-transfection cells were either incubated for 20% O_2 or 0.2% O_2 for 24 hours. All bars represent means ± S.E.M. of the independent experiments performed in quadruplicates. **(C)** Reporter gene assay using the indicated constructs in A, overexpressing STAT3 or USF-2 **(D)** together with PHD hydroxylation-insensitive HIF-1 and HIF-2 constructs. Bars represent means of one experiment in quadruplicates.

All transcription factors shown in Fig. 4B were found to bind by ChIP-sequencing in the *PAG1* enhancer element surrounding the HRE. The mutated enhancer and the non-mutated element were used to control the assay and to set the basal level of hypoxic induction. With overexpression and potential binding of the factors a stronger induction was expected, dependent on the interaction via HIF on the protein level or direct DNA-binding close to the HRE in an HIF-independent manner. No super induction of the signal could be observed in any transcription factor overexpression. Assays overexpressing these factors in combination with HIF-α constructs, having two proline mutations in the oxygen degradable domain (ODD) and therefore being PHD hydroxylation insensitive and the PAG1 wild type construct in normal oxygen and hypoxic conditions was a test, if it is possible to drive the response to one isoform or the other (Fig. 4C and D). No striking difference occurred in the HIF and STAT3 or USF-2 overexpressed samples to the control samples.

Both HIFs bind the core HRE sequence, but further studies on this topic revealed, that the N-terminal transactivation domain (N-TAD) is responsible for the gene activation. To investigate this issue in the case of PAG1, we made use of special HIF constructs, where the HIF-1 DNA binding domain was fused to the N-TAD of HIF-2α and vice versa (Fig. 5A). Hypoxic treatment or overexpression of both HIF N-TAD swapped constructs are able to induce activity, independent of the used promoter. No response could be observed in the mutated construct (Fig. 5A, B).

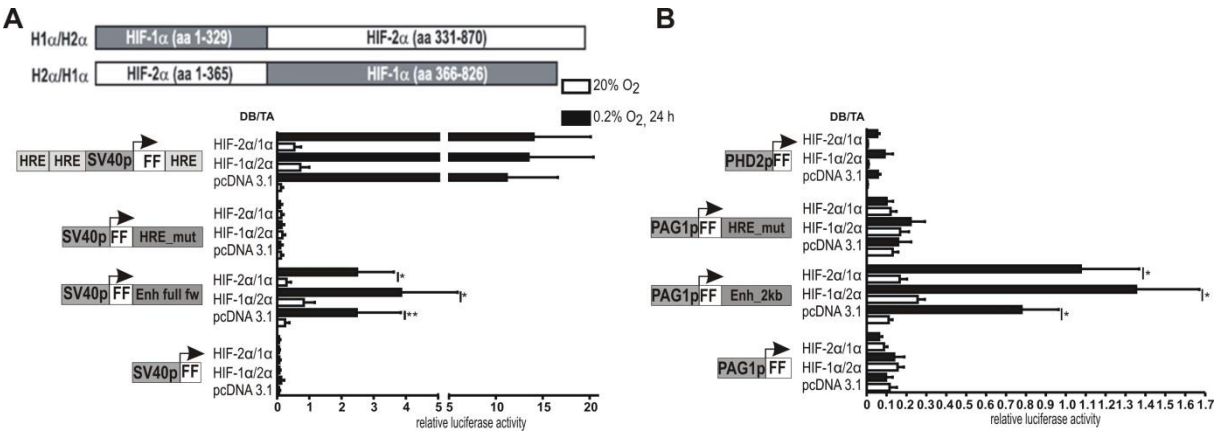

Fig. 5: Discrimination of a potential HIF isoform specificity

(A) Scheme of HIF-hybrid constructs with swapped N-terminal transactivation domains. Reporter gene assays using the indicated constructs transiently transfected in HeLa cells. 24 hours post-transfection cells were either subjected to 20% O_2 or 0.2% O_2 for 24 hours. **(B)** Reporter gene assay with the indicated constructs performed as described in A. All bars represent means ± S.E.M. of the three independent experiments performed in quadruplicates.

The HIF isoform dependency is also dependent on the cell type investigated, as already reported in literature. To see, if we can clarify a potential dependency, we chose two different cell lines HeLa and MDA-MB-231. In both cell lines we created stable single HIF-1α, HIF-2α or double isoform knockdowns to investigate the PAG1 induction in these cells (Fig.6).

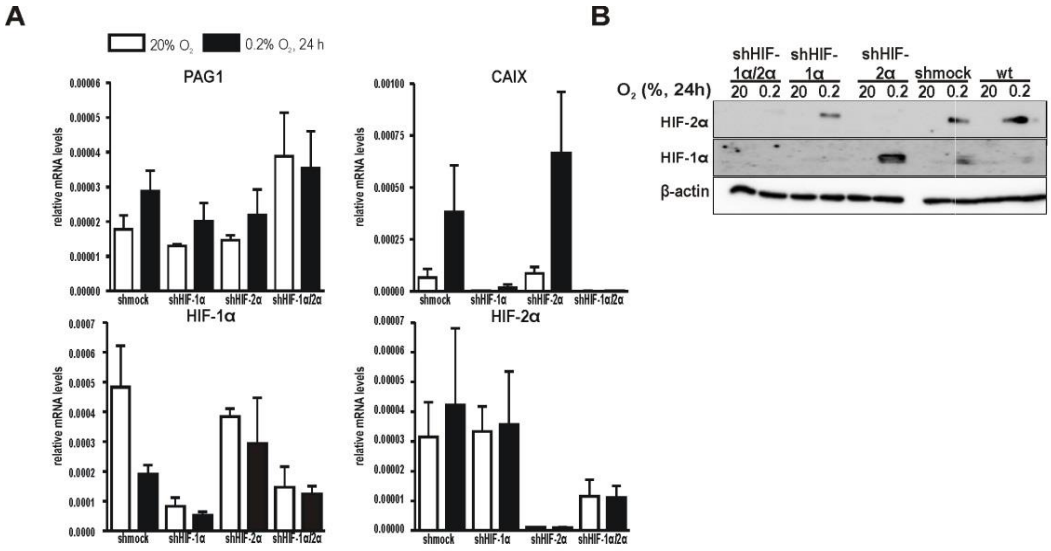

Fig. 6: Assement of HIF-α isoform specifity of PAG1 in MDA-MB-231 cells

(A) MDA-MB-231 shcontrol or HIF knockdowns were exposed to 20% O_2 or 0.2% O_2 for 24 hours. PAG1, CAIX, HIF-1α and HIF-2α transcript levels were analysed by quantitative reverse-transcriptase RT-qPCR. All data shown are means ± S.E.M. of three independent experiments normalised to β-actin values. **(B)** Cells were treated as described in (A). Protein extracts were made using a combined high salt, SDS lysis and analysed by Immunoblotting for HIF-2α, HIF-1α, PAG1 and β-actin.

Analysis of the mRNA levels in the HIF-α knockdown derived from MDA-MB-231 reveal, that PAG1 is inducible from both isoforms, since in the single knockdown a hypoxic induction in either the HIF-1 or HIF-2 knockdown is visible (Fig.6A).

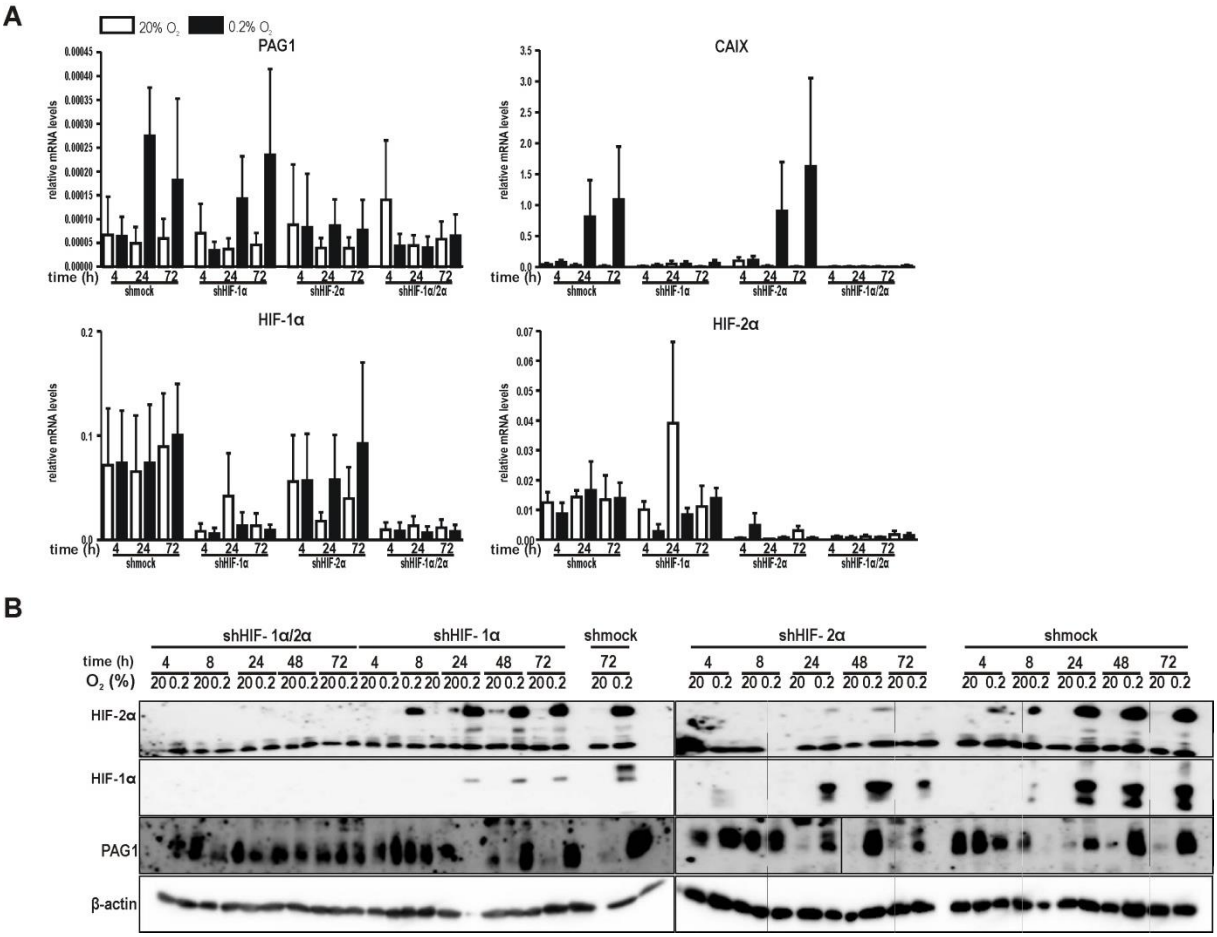

Fig.7: PAG1 hypoxic regulation is dependent on both HIF isoforms in HeLa

(A) HeLa derived shmock, shHIF-1α, shHIF-2α and shHIF-1/2α cells were cultured at either 20% O_2 or 0.2% O_2 for the indicated time points. **(B)** HeLa shmock, shHIF-1α, shHIF-2α and shHIF-1/2α cells were cultured at either 20% O_2 or 0.2% O_2 for the indicated time points. Protein extracts were made using a combined high salt, SDS lysis and analysed by Immunoblotting for HIF-2α, HIF-1α, PAG1 and β-actin.

HIF-1α and HIF-2α display differential roles in function and stabilisation during the course of hypoxia, leading to differential activation of hypoxic target genes. To cover a range from short hypoxic to long term hypoxic exposure, we performed kinetics with the HeLa HIF-knockdowns to investigate a potential effect on PAG1 activation (Fig.7 A+B). PAG1 is up-regulated on the mRNA level in the control cells, as well as in the single HIF-knockdown from 24 hours up 72 hours of hypoxia. No induction is visible anymore in the double HIF knockdown, showing that here a dependency on both isoforms. CAIX, HIF-1α and HIF-2α mRNA levels were measured as control for hypoxic exposure and to control efficient knockdown maintenance during the kinetics. To compare the levels of protein induction and to narrow down, when PAG1 is up-regulated, we performed also protein kinetics with the HeLa HIF knockdown cells, including an additional short hypoxia time point of 8 hours. The results are in line with the mRNA results, showing an up-regulation of PAG1 at 24 hours in the shHIF-1, shHIF-2 and shmock cells. No induction at any time point is visible in the double knockdown cells.

Discussion

We could show that the enhancer element is differentially methylated in between cell lines and in different oxygen conditions. Furthermore both HIF N-TADs are able to induce a response in a dual-luciferase approach, although the signal in the HIF-2α swapped construct are higher. These results are in line with the HIF-α isoform dependency found in HeLa and MDA-MB-231 cells, showing that PAG1 is dependent on both HIF-1α and HIF-2α, but with a relatively late induction in hypoxia (after 24 hours).

The transcription overexpression assay did not bring the expected result. However, not all factors binding in the element were tested, these transcription factors should be included into further analysis. Furthermore a redesign of the array setup should be taken into consideration.

4.3. Role of hypoxia-inducible PAG1 in regulation of Src

Material and Methods

HeLa and MDA-MB-231 cells were cultured as described previously (see manuscript). Starvation experiments were performed in DMEM without FCS. shPAG1 knockdown cells were created with expression vectors encoding short hairpin RNA sequences targeting human PAG1 and a non-targeting control (further referred as mock control) driven under the control of a U6 promoter in a pKLO.1 puromycin containing vector were purchased by Sigma.

pLKO.1 puro	shhumanPAG1_69 (TRCN00000123269)
pLKO.1 puro	shhumanPAG1_70 (TRCN00000123270)
pLKO.1 puro	shhumanPAG1_71 (TRCN00000123271)
pLKO.1 puro	shhumanPAG1_72 (TRCN00000123272)

Lentiviral particles were produced in HEK293T cells, using the Vira-Power Lentiviral expression vector system according to Manufacturer's instructions (Invitrogen). For the creation of shPAG1 knockdown cells HeLa and MDA-MB-231 cells were infected with lentiviral particles containing one of the 4 different shPAG1 shRNAs. Pools of clones were selected with 10 µg and 4 µg puromycin respectively.

The ability of cells to form colonies was assed as followed. 200 cells were plated in triplicates in a 24 well dish in medium as described before. Cells were incubated for 5 days in either 20% O_2 or 0.2% O_2. Cells were washed twice with cold PBS and fixed with 100% Methanol for 10 minutes. For visualisation of the colonies, cells were stained with 0.005% Crystal Violet in 25% Methanol for 10 minutes and washed with water to remove excess dye. Plates were dried at room temperature and colonies were manually counted under the microscope.

For determination of the proliferation rate, cells were plated with a starting number of approximately 150.000 cells in a 96 well plate. Assay was performed as described elsewhere (1).

The cellular ability of cells to grow anchorage independent was assessed with soft agar colony formation assay. The bottom layer was prepared with a 50:50 mixture of 2x DMEM (Sigma) medium and 2% low melting agarose (Sigma). 5000 cells were mixed in the top layer composed of a 50:50 mixture 2x DMEM medium and 0.8% low melting agarose in triplicates in a 24 well plate. Top layer was covered with DMEM medium to prevent drying. After incubation for 14 days either in 20% O_2 or 0.2% O_2 the soft agar was washed with PBS and colonies were visualised by staining with 0.005% Crystal Violet in 25% Methanol and washed with water to remove excess dye. Colonies were manually counted under the microscope.

Results

The Src signalling pathway is induced by a variety of different stimuli, including growth factors. To investigate a potential influence of PAG1 on Src signalling, we chose two different cell lines, the high invasive breast cancer cell line MDA-MB-231 with high basal PAG1 expression and low hypoxic induction levels and the cervical cancer cell line HeLa displaying a low basal PAG1 expression level, but a high hypoxic induction of the gene (see 3.). We compared the Src activation upon stimulation of

the pathway after pre-stavation of the cells for 16 hours in medium containing no FCS, to cells cultured in 10% FCS containing medium in normoxia and 24 hours hypoxia. To investigate the kinetics of the Src Tyr 419, FCS was added at different timepoints (Fig.1).

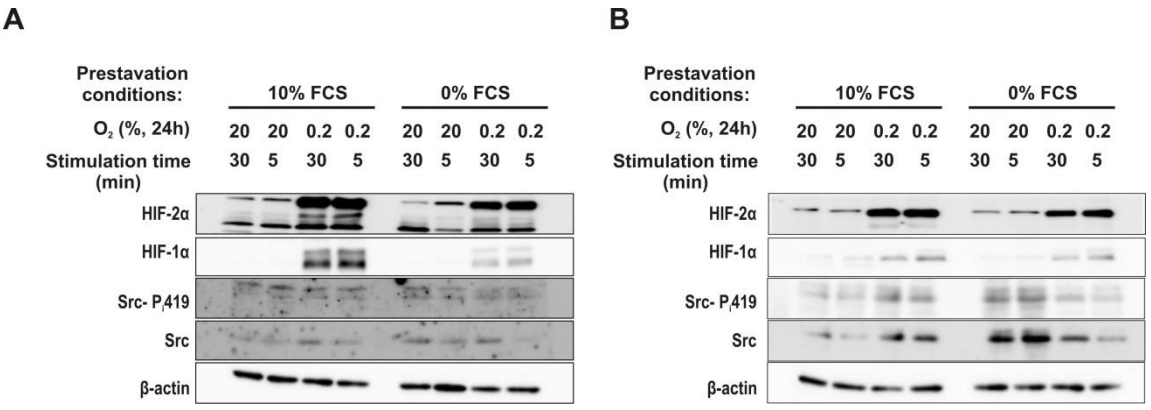

Fig.1: FCS stimulation does not lead to increased Src kinase activity

(A) MDA-MB-231 cells were cultured in DMEM with or without FCS for 16 hours, then subjected to either 20% O_2 or 0.2% O_2 for 24 hours and treated with FCS for the indicated time prior harvesting. Protein extracts were made using a combined high salt, SDS lysis and analysed by immunoblotting for HIF-2α, HIF-1α, PAG1, Src- P_i 419, total Src and β-actin.

(B) HeLa wild type cells were treated as indicated in (A).

We could not observe any striking difference in between Src-P_i 419 levels in serum stimulated and control samples neither in normoxia or hypoxia. Next we created stable shPAG1 knockdowns in these cell lines to investigate the role of PAG1 as a potential regulator of Src. From four different shPAG1 RNAs transfected in HeLa cells (Fig.2A) and two different ones in MDA-MB-231 (Fig.2B), we chose the pool of clones with the best knockdown efficiency, hairpin #72 for HeLa and #70 for MDA-MB-231, to perform functional experiments. Miss-regulation of the Src kinase regulation scaffold due to PAG1 knockdown should lead to increased proliferation, survival and a more aggressive phenotype with transformation features resulting in the ability to form colonies at low cell density and to grow anchorage independent.

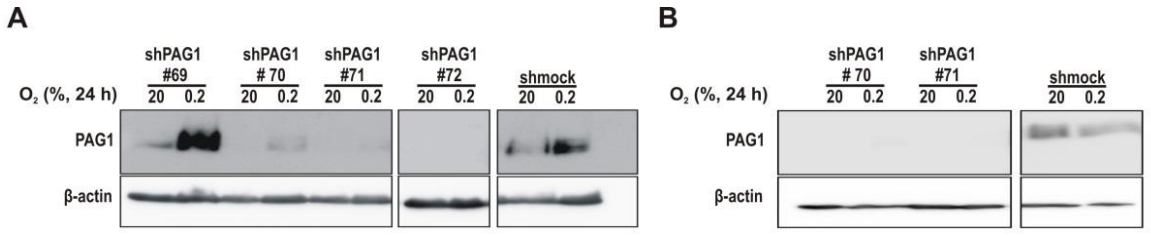

Fig.2: Creation of stable shPAG1 knockdowns in HeLa and MDA-MB-231 cells

(A) HeLa wild type cells were infected with lentiviral particles containing the indicated shRNA against PAG1 or against a non-targeting control (shmock). Pools of puromycin selected clones were subjected to either 20% O_2 or 0.2% O_2 for 24 hours. Protein extracts were made using a combined high salt, SDS lysis and analysed by Immunoblotting for PAG1 and β-actin.

(B) MDA-MB-231 wild type cells were treated as described in (A).

We could not observe any difference in proliferation, colony formation or anchorage-independent growth in HeLa PAG1 knockdown cells compared to control cells, cultured in normoxia or hypoxia. In contrast we observed a significant proliferation reduction in MDA-MB-231 PAG1 knockdown cells

compared to control cells, when cultured in hypoxia (Fig.3D). Although the no noticeable difference can be observed in low densitiy colony formation, we found a PAG1 dependent reduction of anchorage-independent growth, independent of the culturing conditions (Fig.3F). This effect is clearly PAG1 and not hypoxia dependent.

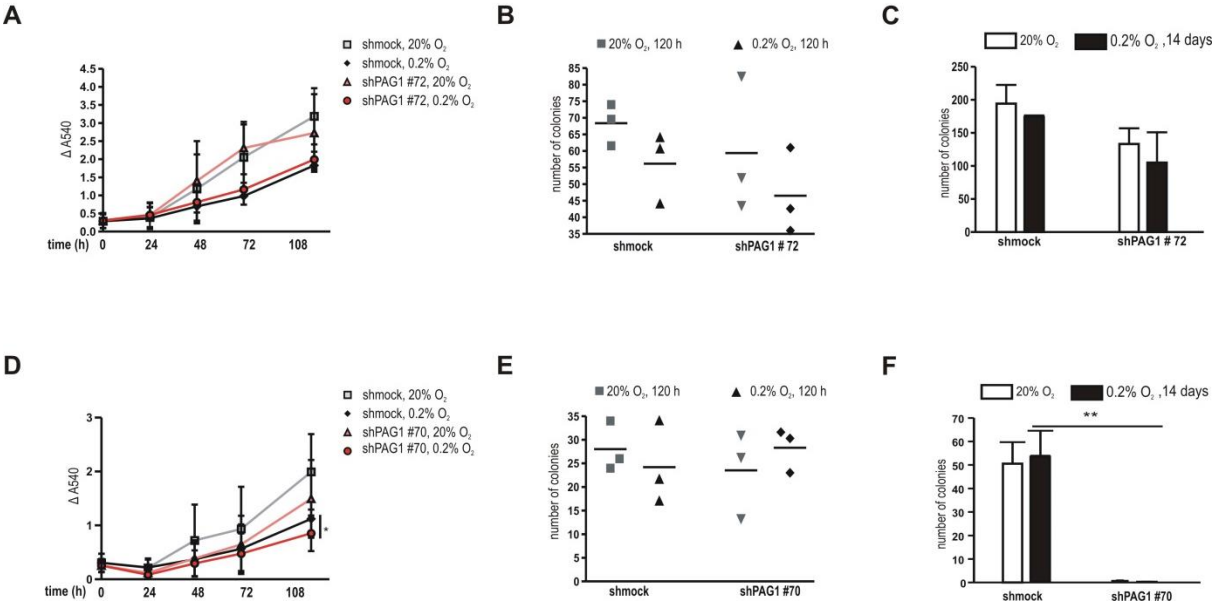

Fig.3: Role of PAG1 in proliferation and transformation features

(A) Proliferation of shmock and shPAG1 #72 HeLa cells kept under normoxic or hypoxic conditions for up to 5 days was determined by SRB assay.

(B) Low-density colony formation of shmock and shPAG1 #72 HeLa cells cultured under normoxic or hypoxic conditions for 5 days.

(C) Anchorage-independent colony formation of shmock and shPAG1 #72 HeLa cells cultured in soft agar under normoxic or hypoxic conditions for 14 days.

(D) MDA-MB-231 shmock and shPAG1 #70 cells were processed as described in (A).

(E) MDA-MB-231 shmock and shPAG1 #70 cells were treated as described in (B).

(F) MDA-MB-231 shmock and shPAG1 #70 cells were analysed as described in (C).

As neither the stimulation of the Src kinase pathway, nor any functional analysis of PAG1 knockdown cells could bring a final proof, that PAG1 is regulating the Src kinase activity in these cells, a potential interaction on the molecular levels was investigated. Additionally it would give indication for an additional layer of Src regulation in hypoxia, that the hypoxic up-regulation of PAG1 reduces Src kinase activity.

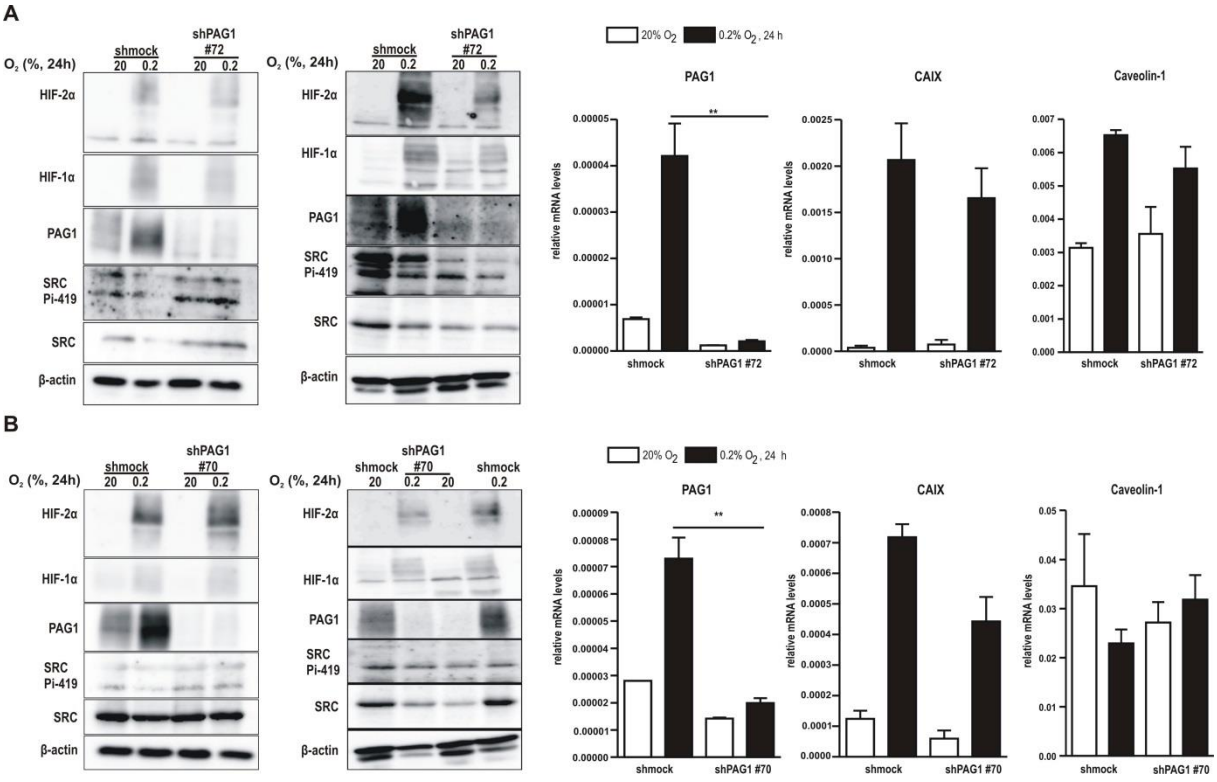

Fig.4: Src activation is independent of hypoxic PAG1 up-regulation

(A) shmock and shPAG1 #72 HeLa pools of clones were subjected to either 20% O_2 or 0.2% O_2 for 24 hours. Protein extracts were made using a combined high salt, SDS lysis and analysed by Immunoblotting for HIF-2α, HIF-1α, Src P_i-419, total Src, PAG1 and β-actin. PAG1, CAIX, Caveolin-1 transcript levels were analysis by quantitative reverse-transcriptase RT-qPCR in shmock and shPAG1 #72, either cultured at 20% O_2 or 0.2% O_2 for 24 hours. All data shown are means ± S.E.M. of three independent experiments normalised to β- actin values.

(B) shmock and shPAG1# 70 MDA-MB-231 cells were processed as described in (B).

In both cell lines a reduction of active SRC-P_i419 phosporylation could be observed (Fig. 4A and B left panel). These results were not reproducible.

Discussion

The non-reproducible results, obtained in the last experiments (Fig.4), lead more to the conclusion that another TRAP member or another Csk adaptor can compensate for the loss of PAG1 at least in HeLa cells. A potential candidate is Caveolin-1, also a HIF target and up-regulated upon hypoxia (Fig. 4A)

The experiments performed with MDA-MB-231 cells lead to the conclusion that PAG1 is not directly regulating Src signalling, but is implicated in important cellular functions.

References

1. Vichai, V. and Kirtikara, K. (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc*, **1**, 1112-1116.

5. Conclusion

5.1. The HIF heterodimer and its target genes

The imbalance between oxygen delivery and consumption causes a decrease in of oxygen availability in the tissue. This lack leads to the stabilisation of the HIF-α isoforms, managing the cellular response to the low oxygen partial pressure. This described event here has an essential influence in pathological diseases like cancer, arteriosclerosis, stroke, ischemia and inflammation (1-3).

After a decade of intensive research on the HIF transcriptome, revealed over 100 HIF dependent hypoxia-regulated genes have been found. Starting from the detection of mRNA changes with Northern Blot analysis, such as for VEGF the technical advantages supported the identification of more stimulus dependent HIF target genes (4). The gold standard today are gene expression analysis by RNA-sequencing or cDNA microarrays, which allows the identification of a huge set of gene expression changes upon different treatments *in vitro* and *in vivo* (5). In the case of hypoxia, lots of different arrays, already varying concerning the experimental setup, cellular models were used to study the changes between normoxic and hypoxic gene expression. We performed RNA microarray analysis to compare the expression of genes in HeLa cells exposed to normoxic and hypoxic conditions. Besides the very well established HIF target genes such as *Ca9*, *EGNL3* and *NRGD1*, which showed a 74.76, a 7.464 and an 18.38 fold induction respectively, in hypoxia. Two hypoxia induced genes *ANGPTL4* and *SPAG4*, showed a fold increase of 15.22 and 11.89 respectively (see 3.). Later, two independent studies confirmed *SPAG4* as novel HIF target gene (6,7). Also *ANGPTL4* was repeatedly confirmed as an integrated target of HIF and peroxisome proliferator-activated receptor (PPARβ/δ) in HUVEC cells (8). The independent confirmation of these target genes in other cell lines than HeLa cells underlines the quality of our array. In this experiment we identified the transmembrane adaptor protein *PAG1*, also named *Cbp*, as a novel hypoxia-induced gene with a 2.9 fold induction. In comparison to the other new genes, the hypoxic fold induction is low. Due the best of our knowledge it is the only microarray analysis comparing normoxic and hypoxic cell line samples or other HIF-dependent conditions, where *PAG1* is reported to be up-regulated. This effect of increased mRNA abundance after exposure to hypoxia could be verified in a set of different cancer cell lines (see 3. and 4.1.).

Hypoxia has different effects on gene transcription. Besides direct binding of the HIF heterodimer in regulatory regions of the gene (9), HIF independent gene regulation, so just an indirect effect due to the lack of oxygen, also the mRNA stability can be increased. This cannot be distinguished by microarray analysis, which only shows the quantitative amounts of mRNA present in the samples. Different studies show for VEGF mRNA, that under hypoxia the transcript half-life increased from 43 ± 6 min to 106 ± 9 min, due to posttranscriptional modifications (10). The opposite could also be observed, so hypoxia decreased the mRNA stability of nitric oxide synthase (eNOS), the mitochondrial manganese-containing superoxide dismutase (Mn-SOD) and the cytosolic copper & zinc-containing superoxide dismutase (Cu, ZN-SOD) (11,12). Based on these studies we analysed if the increased mRNA levels are directly translated into protein (see 3. and 4.1.). We could observe a clear correlation between hypoxic mRNA increase and up-regulated protein levels.

The PAG1 protein is also regulated by hypoxia is a broad range of different cancer cell lines. We show that this gene is regulated by hypoxia in a broad panel of hematopoetic system derived cancer cell lines and a broad range of cancer cell lines of different tissue origin (see 3. and 4.1.). *PAG1* is one of the few genes, which is broadly up-regulated in cells, similarly to *CA9* or *EGLN1* and *EGLN3*. In contrast, hypoxic regulation of some genes is tightly restricted to certain tissues, like the hormones leptin, produced from the adipose tissue and the placenta or erythropoietin, produced in the fetal liver and in adults mainly from the kidney, but also in the brain, the liver and in testis (13-16). Although PAG1 is not hypoxically regulated in some cell lines, such as MDA-MB-468 and A459, in most other cell lines tested we found an up-regulation of PAG1. In addition we could translate this tissue culture restricted phenomenon *in vivo*. Transcript level measurements of mice subjected to inspiratory hypoxia, showed that Pag1 is up-regulated in different tissues, including heart, liver, lungs, spleen and kidney (see 3.). Here we could bridge the first gap from cell culture to *in vivo* conditions. Among the huge amount of hypoxia regulated genes, only some are also found *in vivo* or used as tumor hypoxia markers. Intrinsic tumor hypoxia markers are seen as a reliable detector, whereas exogenous markers have immense drawbacks due to their application method. 15 different genes were found in serum and tissue samples out of 213 different studies from different cancers. These genes belong to different groups. Transcription factors like HIF-1α, NF-κB and AP-1 can be found in immunohistochemistry in different tumor samples. Besides metabolic adaptors like CAIX, GLUT1 and LDHA could be found in tissue samples and additionally LDHA in serum samples respectively. Also VEGF, EPO, PAI-1, BNIP3 and LOX were confirmed and therefore regarded as tumor hypoxia markers (17). PAG1 increased mRNA expression in several human cancer cell line and hypoxic mouse tissue samples was found in the current work. Further investigations should be performed on correlation studies on human cancer tissue samples, if PAG1 can also function as hypoxic tumor marker. *In silico* cancer correlation studies, using the R2 database, confirmed a correlation of *PAG1* and *HIF1A* in tumor prostate samples. In mixed colon cancer samples a correlation between *EPAS1* and *PAG1* could be found (data not shown). It should be noted that these analysis are based on cancer tissue microarray datasets, so also an immunohistochemical confirmation would be necessary. This analysis could also fill a gap of this study, investigating if also in hypoxic human tumor tissue samples PAG1 is up-regulated. These kind of studies were performed for different new HIF target genes or HIF interaction partners like AREG and ETV4 (18,19).

5.2. Identification of a novel distal hypoxia-activated enhancer element regulating *PAG1* expression

Reduced oxygen conditions (hypoxia) lead to cellular stabilisation of the oxygen-sensitive HIF-α subunits. These protein shuttle to the nucleus and form a heterodimer with ARNT and act as transcription factor with binding its core HRE -RCGTG- to activate its target genes, essential for the hypoxic adaptation processes (9). HIF-α binds the -NAC- part of the sequence, whereas ARNT binds the -GTG- part of the consensus HRE (20). Technological advantages in the analysis of protein-DNA interaction and sequencing technology eased investigations on genome-wide transcription factor

profiling. Chromatin immunoprecipitation (ChIP) coupled with DNA-sequencing or with DNA-microarray (ChIP-on-chip) are used to identify protein-bound DNA sequences (21)**.** Up to now five different studies have analysed the genome-wide HIF-α binding profile in different cell lines, and a varying number of binding sites from 377 to 2060 (22-26). The quality of these analyses are more reliable than *in silico* analysis, where only an algorhytmical based prediction of HIF binding site could be made (27,28). We used the experimental output of the five genome-wide studies to have a first indication for identifying the HIF binding site, responsible for the *PAG1* hypoxic induction. We found in two different pan-genomic studies a HIF binding site laying 85 kb upstream of the *PAG1* transcriptional start site (TSS). This site was found to be a HIF-2α binding site in MCF-7 cells and a HIF-1α binding site in HUVEC cells (26,29). We further characterized this HIF binding site in a 317 bp fragment by reportergene assays, which revealed a hypoxia and HIF dependent response (see 3. and 4.2). Pan-genomic correlation of HIF binding studies and chromatin structure modification studies enables to describe an epigenetic environment of HIF binding sites. Most of these sites overlap either with a DNAseI hypersensitivity cluster or with Nucleosome-sequencing data under normoxic conditions (23,29). Furthermore, for distal regulatory elements, certain chromatin modifications are reported to help to identify these regions. The understanding that enhancers share common chromatin features, led to a revolution in the ability to identify these regions in a genome-wide, cell type specific and reliable manner. Enhancers can be divided into active, primed and poised elements with common and specific features. All of them share the H3K4me1, the first modification globally linked to distal regulatory elements. In the same study Heintzmann and coworkers identified the H3K4me3 as a specific mark for promoter regions (30). Active enhancers show in addition a DNAseI hypersensitivity site, H3K27ac, RNA pol II binding. Primed and poised enhancers lack these features (31). *In silico* analysis and ChIP-qPCR experiments displayed all the chromatin features of an active enhancer element. We could rule out that the *PAG1* enhancer element, where the HRE is inside, is a poised or only an primed enhancer. We also performed ChIP experiments for H3K4me3 in this region. Although this modification is normally known as promoter feature it also appears in enhancers. However the ratio between H3K4me1 and H3K4me3 is important to distuingish enhancers from promoters. As we show also the enhancer has a higher H3K4me1 levels compared to the H3K4me3. In addition we verfied the binding of HIF-1, HIF-2 and ARNT by the same method. We could also detect the binding of the cofactor p300. We identified a distal hypoxia inducible enhancer element regulating the gene expression of *PAG1* (see 3. and 4.2.).

The core consensus HRE sequence -RCGTG- is essential for HIF DNA binding and target gene activation. On the layer of epigenetic DNA modification HIF transcriptional activity can also be regulated. HIF and ARNT belong to the methylation sensitive transcription factors, where the binding is disabled when a 5'-Methylcytosine is present in the DNA binding sequence. The 5'-Methylresidue is reaching into the major groove of the DNA, blocking HIF-α binding, which is contacting the DNA in the same area (32). A study from Denizot and colleagues, that carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression is increased due to demethylation of the HIF binding site in the promoter during hypoxia or HIF stabilising agents. Based on their bisulfide sequencing results, they also revealed that the HRE is demethylated during HIF stabilisation and the methylation is

decreased in mice fed with methyldonor-deficient diet (33). Till today a variety of hypoxia target gene expression studies only show, that DNA demethylation at the HRE is required for effective HIF target gene activation (34,35). Changes in DNA methylation induced by hypoxia are reported for human colorectal and melanoma cell lines (36). In human hepatoma cell lines hypoxia induces genomic DNA demethylation through the direct activation of Methionine adenosyltransferase 2A (MAT2A), a direct HIF-1 target gene. This enzyme maintains the homeostasis of S-adenosylmethionine (SAM), a critical marker for genomic methylation status (37). Our finding for the methylation status of the PAG1 enhancer HRE show a higher methylation in normal oxygen conditions compared to hypoxia (see 4.2.). This finding is supported by the aforementioned studies, as HIF has to get access to its binding site for efficient target gene activation.

The distance of 85 kb between TSS and the *PAG1* HRE is one of the most remote HREs (see 3.). This mode of hypoxic gene regulation was already intensively studied for different genes. The longest known gene is *EPO* regulated by an hypoxic enhancer located at 3' of the gene (9). Later analysis revealed a 5' HRE, potentially contributing to the kidney specific *EPO* induction (38). The most distal HRE enhancer element was reported to regulate the *CCND1* expression, encoding Cyclin D1, with a distance of 200 kb (24). However it seems that the enhancer element regulates only one gene, as in our case *PAG1*. Evidence for this notion is that *FABP5*, laying upstream of the hypoxic enhancer element, shows no regulation in hypoxia (see 3.). However, we show here for the first time, that the other flanking gene is not regulated by the hypoxic enhancer element (see 3.).

Chromatin looping inbetween regulatory regions can be detected with the help of fluorescence in situ hybridization (FISH) or chromatin conformation capture (3C) technology (39). Genome-wide 3C analysis for different transciption factors were already peformed. A genome-wide study in IMR90 cells, investigating the long-range interaction of enhancers bound by the p65 subunit of NF-κB found pre-existing chromatin loops between the promoters of NF-κB responsive genes and their corresponding enhancers (40). The same chromatin architecture was found for looping induced by p53, forkhead box O3 (FOXO3) and the glucocorticoid receptor (GR) (41-43). Including our research on the *PAG1* enhancer, there are only three studies investigating this issue. Mimura and colleagues performed research on the HIF-1α dependent gene activation of *SLC2A3*, encoding for Glut 3. They found two enhancer elements, enhancer 1 located -35 kb upstream of the TSS, enhancer 2 lays -24 kb upstream. Both are required for full hypoxic activation. Based on 3C-ChIP experiment results, using a HIF-1 antibody, they found that both enhancers pre-formed a chromatin loop already in normoxia. But both enhancers only interact with each other in hypoxia, to fully maintain increased gene expression (26). The second study performed by Schödel and colleagues investigated the chromatin looping of the *CCND1* gene, encoding Cyclin D1. In their comparable analysis they showed chromatin interaction between the -200 kb distal element with the gene promoter only the pVHL defective, constitutively HIF-2α expressing 786-O cells. This interaction could not be found in hypoxic, pVHL expressing MCF-7 cells (24). This result suggests, besides their claimed cell type specific effect, that the HIF heterodimer binding in distal elements is essential for the development of an enhancer-promoter interaction chromatin loop. Based on this aforementioned knowledge we performed 3C analysis on different cell types and cell lines, to clarify if the regulatory chromatin loop is HIF dependent or if this loop is

preformed, as showns for other transcription factors. With our experimental setup we found the formation of regulatory loops in and surrounding the enhancer element. Furthermore we saw the building of regulatory loops between the PAG1 enhancer, the promoter and even in the 1. Intron of the 5'UTR (see 3.). These results are quite expected, as it already very well reported that this region for genes with a long 5'UTR, like for PAG1, has a regulatory function in gene expression (44). So also a chromatin loop reaching into the 5'UTR was expected. These chromatin loops were formed already in normoxia, with minor changes in strength in hypoxia (see 3.). This could be caused potentially due to RNApolII or other unkown transciption factor binding, leading to this variation. But the overall picture shows a preformed looping as described for the aforementioned transcription factors. We found this chromatin structure in cell lines, where we see a HIF-dependent PAG1 regulation. The next aim of this study was to clarify if HIF itself hav to be present to maintain the looping. To clarify this issue we compared the chromatin structure of normoxic and hypoxic shmock and shHIF-1α/2α double knockdown MCF-7 cells. Also there we found the same loops independent of the presence of HIF already in normoxia (see 3.). Furthermore we evaluated, if the presence of a HRE in the enhancer element has a influence on the regulatory chromatin architecture. TALEN-mediated destruction of the HRE in HeLa and MCF-7 cells provided a powerfull tool to investigate this topic. The chromatin architecture is maintained independent of the presence of the HRE (see 3.).

Taking all these findings together we can conclude here, that chromatin loops inbetween regulatory regions are preformed in a cell line specific, gene expression dependent and main transcription factor independent manner. With our study we show for the first time a correlation between PAG1 hypoxic gene expression and we give evidence that HIF or the presence of a HRE is not the main factor for the formation of a regulatory chromatin looping. So HIFs belongs to the same group as NF-κB, p53, FOXO3 and GR, which already form pre-existing chromatin loops to maintain a fast and cell line specific adaptation to external stimuli (41-43). To give a final proof for the currently described findings, a genome-wide chromatin interaction and expression study for the HIF transcriptome in different cell lines would be essential. This study would give a broader insight in HIF dependent gene regulation. Because we lack further analysis on the regulatory chromatin architecture of PAG1 in a cell line, where is not hypoxically regulated, suggesting that the regulatory chromatin interaction should not be present there. Furthermore, like the studies from Schödel and Mimuara and coworkers, we also did not investigate if our enhancer element is only *cis* or also *trans* acting. This could be clarified by genome-wide 4C or 5C anaylsis comapring normoxic and hypoxic cells (45).

5.3. The *PAG1* enhancer element as an integrated transcription factor platform

A central feature of enhancers is their ability to function as transcription factor binding sites. In fact, these DNA elements contain multiple recognition sites for different classes of transcription factors. These proteins recognize a 6-12 bp long specific DNA sequence. Different modes of gene regulation are described for this class of proteins. Either they are contributing via direct DNA-protein binding or indirectly via binding to additional transcription factors (31). Based on the ENCODE database, 37 different transcription factors are binding in the *PAG1* enhancer element (see 3.).

Among these factors are also the so called pioneer factors FOXA1 and FOXA2 (46). In particular, FOXA1 was shown, that this factor has the capacity to access its binding site in nucleosomal DNA, decompact chromatin and reposites nucleosomes of target enhancers *in vitro* and *in vivo* (47). FOXA2 is also an important factor for chromatin remodelling (48). As these factors are important for opening enhancer regions to give access to other factors, this is an additional characteristics of an enhancer element. Besides pioneer factors also p300 was found to bind in the *PAG1* enhancer region. This factor displays histone acetyltransferase actitivity and one its substrates is H3K27 (49). The chromatin modification H3K27ac is a manner to distinguish active enhancers from poised enhancers (50). Besides this feature, p300 is also a very well known cofactor for other factors, including HIF-1α and HIF-2α (51). Besides the aforementioned factors, a huge variety of transcription factors were found to bind in this region. Moreover one repressor was present: RCOR1. The Rest corepressor 1 was first identified as a transcriptional repressor of neurnal genes in non-neuronal cells (52). The rest of the factors participate in gene activation. Among these are also a set of cofactors, shown to interact with either HIF-1α or HIF-2α, like Sp1, Fos, Jun, STAT3 and MYC (19,53-57). With our transcription factor overexpressing screen we aimed to evaluate if some of the already reported factors have an influence on the enhancer activity (see 4.2.). As none of the tested factors showed reliable results, the assay setup should be redefined. Another explanation could be that none of these factors, which were found to bind in the enhancer region in different cell lines, are not binding in the cell model used, HeLa. As STAT3, only interacting with HIF-1α was found to bind in the *PAG1* enhancer we tested this factor together with USF2, the cofactor interacting solely with HIF-2α (58). This research line did also not bring any conclusive result (See 4.2.). Of important note is the fact that these ChIP experiments were performed in normoxic conditions, whereas hypoxia could lead to a change of transcription factor binding in this area. In addition, the transcription factor binding profile is cell line-specific, so maybe some of these factors exclusively bind in the enhancer region in these cell lines and not in other ones. On the other hand we tested only a small subfraction of the factors displayed. Further investigations including all transcription factors with a revised setup should be taken into consideration. The synthetic screen of Wollenick and colleagues showed, that it is possible to identify novel HIF cofactors with their experimental conditions, in their case the *PHD2* promoter (19).

5.4. HIF isoform specifity of *PAG1* hypoxic response

The central role of cellular hypoxic regulation is mediated by the transcription factor hypoxia-inducible factor (HIF). Analysis of HIF-DNA interactions defined the core -RCGTG-, where HIF is binding. More complexity is added by the existence of multiple isoforms, with the best studied ones HIF-1 and HIF-2α. These have similar domain architectures and both bind to the core HRE consensus sequence. Additionally they are able to activate HRE-linked reporter genes. However both display different developemental phenotypes *in vivo*. Although the tissue specific expression could contribute to this effect, a variety of studies revealed that both isoforms have a pool of common and distinct transcriptional targets (59).

PAG1 is a hypoxia up-regulated gene in a set of cancer cell lines. Our analysis showed that this gene in regulated by an enhancer element, containing an HRE, 85 kb up-stream of the TSS. To investigate, which isoform drives the hypoxic up-regulation we made use of already reported analysis. Studies investigating this topic revealed that both HIF-ARNT heterodimers bind to the HRE of target genes, but the N-terminal transactivation domain is important for specific target gene activation. Analysis with heterologous reportergene assays using HIF-1α or HIF-2α N-TAD deletion constructs revealed in the case of CITED2 and PAI-1, that this domain is important for target gene activation (60). In another analysis from Stiehl and coworkers, they tried to identify which isoform is important for hypoxic *AREG* activation. In their hands none of the constructs induce the *AREG* promoter (18). In the case of the *PAG1* enhancer element, both domain swapped constructs were able to induce reportergene activtiy in HeLa, but the HIF-2 NTAD showed a stronger induction (see 3.). These result together with the ChIP data in MCF-7 showed occupancy of the HRE with both isoforms, but a higher binding of HIF-2α. In 786-O cells HIF-2 bound to the enhancer HRE, which was quite expected, as these cells lack the HIF-1 isoform (see 3.). Another possibility to identify the HIF-α isoform, is to create stable single HIF-1α, HIF-2α and double knockdowns. We made use of the already reported MCF-7 HIF knockdown cells (18). In addition we created these knockdowns in MDA-MB-231 and HeLa cells (see 3. and (61). Analysis in these cell lines revealed, that indeed in MCF-7 cells and 786-O cells PAG1 is a HIF-2α dependent target. This finding confirms the ChIP analysis (see 3.). In HeLa and MDA-MB-231 the isoform dependeny could not fully be defined. Although the hypoxic induction is completely abrogated in cell lines expressing both HIF-α shRNAs, a reduced induction can be observed in the HIF-2 knockdown cells compared to HIF-1. In addition, anly in late hypoxic timepoints an induction could be observed in HeLa cells (24 hours and later), which would also suggests a HIF-2 dependent regulation (see 4.2.). But this could not be confirmed, at least in these two cell lines. So the results in this case stand in contrast to each other. *PAG1* is direct a HIF-2 target in 786-O and MCF-7 cells, whereas it is more activated upon hypoxic stimulus by both HIF-α isoforms in HeLa and MDA-MB-231 cells. Due to the current model, based on ChIP-sequencing analysis HIF-2 binds to promoter distal HREs, contributing more to tissue specific gene activation, whereas HIF-1 tends to bind rather promoter proximal regulatory elements, maintaing the general gene activation (29).

This issue is complicated by the fact that both factors are normally expressed in most cancer cell lines, which are used to study the molecular mechanisms of hypoxic adaptation (62). As we used the same kind of model to verify the result of our microarray, we could proof that the *PAG1* hypoxic up-regulation is not restricted to HeLa cells. In addition we showed the first time, that our gene of interest is also induced in different organs from mice, exposed to low oxygen (see 3.). Normally most studies investigate their *in vivo* veryfication in tumor tissue with immunohistochemical stainings, showing a correlation between HIF expression and the expression of their studied gene (6,19,63). Our observation regarding the hypoxic induction of *PAG1* could be verified in a more physiological background,showing that in non-tumor tissue the gene is regulated (see 3.).

The identification of the HIF isoform specificity is a huge topic in the hypoxia field. Several studies show, that the regulation by one isoform is extremely cell line dependent . This issue was tried to be explained by the enhanceosome model introduced by Pawlus and Hu. Besides the HIF-α/ARNT

heterodimer the complex consists of different other isoform specific co-activators (64). Since the cancer cell lines derived from different tissue and display a differential setup of transcription factors, therefore the cell type specifity of the HIF enhancosom complex formation should be considered. The regulation of a HIF target gene was mostly only shown in one particular cell line, where one isoform regulates the gene expression (6,7,24). To the best of our knowledge the current work is the first one that expanded the panel of investigated cell lines, to further proof our hypothesis, that *PAG1* is a HIF-2 dependent target gene.

5.5. Modification of regulatory elements and transcription factor binding sites *in vitro* with genome editing

The identification of HREs, regulating the gene activation, is an essential part of hypoxia research. Untill today scientists had only few possibilities to proof that their proposed HRE is the one responsible for gene activation. With the help of Electrophoretic mobility shift assays (EMSA), the binding of HIF-1α to the core HRE sequence was identified. Mutation of conserved consensus -RCGTG- sequence showed an abrogation of the HIF binding in the 3' enhancer region of the *EPO* gene (9). In addition a huge portion of the known HREs and their target genes, was identified with heterologous reporter gene assay or EMSA analysis (20). The DNA sequence of interest was cloned in a reporter gene containing plasmid and analysed. Also here site-directed mutagenesis of the HRE sequence should lead to an abrogation of the hypoxia induced signal (65). We also used this technique to identify our HRE driving the *PAG1* hypoxic response (see 3. and 4.2.). The drawback of these techniques are, that the systems are very artificial and do not reflect the cellular context.

The ability to precisely target and edit the genome of cells and organisms improved over the last 20 years. A huge variety of techniques was developed to edit genomes including adapting components from other species (66). Starting from transposable elements, such as transposons, group II introns and recombinases, which were the first tools to modify genomes (67-69). Although these tools are not able to specifically target a genomic sequence, they are still widely used to create transgenic animals (70). However the possibility to do directed genome editing in cells and organisms was introduced into the field with zinc-finger nucleases (ZFNs). This tool is designed by fused zinc-finger DNA-binding domain with a *FokI* endonuclease (71). Transcription activator-like effector nucleases (TALEN) have the same modular composition like ZFNs. However TALEs are from bacterial origin, the plant pathogen *Xanthomonas sp*. They act as transcription factors, activating specific plant host genes, which are necessary for the pathogen replication and spread. These TALEs, with their modular setup can be used to target a huge variety of genomic sequences, with low off- target effects. Fused to a *FokI* endonuclease, they are able to induce specific double-strand breaks in the sequence of interest (72,73). Two potential outcomes are possible, when the double strand break is repaired. The cellular DNA reparation machinery can fill up the break with a random sequence, also called non-homologues end joining (NHEJ) or with specific sequence from another allele or from a donor plasmid called homologues recombination (HR) (74). Another genome editing tool, based on the bacterial immune system is the clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR

associated genes (Cas), encoding for the Cas9 endonuclease. The recognition site is a short protospacer adjacent motif (PAM), lying inside the genomic DNA, which leads the Cas9 to the specific cleavage site (75).

All these methods have their positives sites and their drawbacks (66). We decided to use for our approach TALEN technology, due to its target sequence flexibility, low cytotoxicity, good tolerance by cells and already intensively studied off-target effects (76). Besides the editing of coding sequences, this technology was already used to modify enhancers. The study from Mendenhall and colleagues described the alteration of enhancer elements in K562 cells, with a TALE construct fused to the lysine-specific demethylase 1 (LSD1) (77). This enzyme is known to remove H3K4 methylation. Webster and colleagues used TALEN-based genome editing to mutate the melanocyte-specific transcription factor, (MITF) transcription factor motif in the MET enhancer element in melanoma cells (78).

In our setup we used two independent TALEN pair constructs to specifically target the -RCGTG-sequence in the core HRE of the *PAG1* enhancer element in MCF-7 and HeLa cells. After creation and selection of single clones by limited dilution and a co-transfected selection marker, followed by a PCR based screen to identify NHEJ in our region of interest, the clones were functionally analysed for *PAG1* hypoxic induction. Surprisingly the positive HeLa clones obtained with the first TALEN pair showed an increased basal PAG1 expression level in normoxia and a complete abrogation of the hypoxic induction. Sequencing analysis of the destroyed HRE in the enhancer revealed that only small deletions of 4-7 bp occurred (see 3.). These results suggest that the enhancer element could potentially act as normoxic repressor of gene transcription, leading to low basal levels in these two cell lines. ARNT is known to bind GTG part of the HIF binding site (20). Besides HIF-α in hypoxia ARNT has also other dimerization partners. This protein heterodimerizes with partners, mediating circadian rhythm gene programs, innate and adaptive immune responses, oxygen-sensing mechanisms and compensate for deleterious environmental exposures. Some contribute to the etiology of human pathologies including cancer because of their effects on cell growth and metabolism (79). One of these transcriptional interactions could potentially bind in normoxic conditions to the enhancer and leading to downregulation of the gene. In hypoxia, the HIF-α subunits compete with this unknown factor and lead to up-regulation of *PAG1*. Due to the NHEJ in the region, the binding is impaired and leads to up-regulation of PAG1 in normoxia. Another explanation for this effect could be that the deletion leads to creation of a new transcription factor binding site. This unknown new factor then leads to up-regulation *PAG1*. *In silico* comparison with the JASPAR database of wild type and mutated sequences did not bring any conclusive results (data not shown). Further investigations could be performed on the influence of other ARNT interactors.

The analysis of the clones treated with the second TALEN pair, showed the expected outcome. Barely any mRNA and protein expression could be observed in the clones obtained from MCF-7 and HeLa cells. This gene knockout phenotype stresses the general importance of this enhancer element, not only in hypoxia, but already in normoxic conditions. Sequence analysis revealed huge deletion of over 20 bp in the HeLa clone and small deletion in combination with a 67 bp insertion in MCF-7 cells (see 3.). These genome modifications could also lead to a loss of transcription activator sites or to the

creation of a new repressor site. Analysis of the chromatin interaction between the enhancer and the promoter revealed an abrogation of interaction in the NHEJ positive clones already in normoxia. Due to our knowledge, it is the first time a study describes with our setup the genomic editing of the binding sequence of one transcription factors and analysed the outcome. Here we could show, that the HIF heterodimer is essential for the hypoxic induction of genes and the abrogation of chromatin loops in the TALEN clones stresses the importance of HIF in hypoxia regulatory elements guidance. We finally are able to give the ultimate proof for a dedicated HRE regulating its target gene in cellular context, highlighting the immense biological relevance of this work.

5.6. PAG1, an ubiqutiously expressed gene with no function?

The function of the phosphoprotein-associated with glycosphingolipid enriched microdomains (PAG1), also named the Csk binding protein (Cbp) was intensively studied, since its discovery in the year 2000 by two different workgroups. They first described it as novel regulator of the Src family kinases (SFKs), acting as a transmembrane adaptor protein for the major regulator of these proteins, via recruiting the c-terminal Src kinase (csk) with tyrosine phosphorylation at its residue 317 to the membrane. They characterised the protein function in rat brain and in its participation in T-cell receptor signalling (80,81). More following up studies showed a role for up-regulated and downregulated PAG1 in cancer, raising another controversial discussion about its role as tumorsupressor or proto-oncogene (82).

With our study we could proof that PAG1 is widely-expressed in different cancer dervied cell lines and up-regulated in most of them upon hypoxia. This finding adds a novel layer of potential PAG1 function (see 3. and 4.1.). We also aimed to find a link between hypoxic up-regulation and a function for PAG1. Focussing on its potential role in cancer, as also in tumors areas of hypoxia occur, we investigated PAG1 in cancer cell lines (see 3. and (83). As mentioned before, Pag1 was shown to only have minor influence in mice brain maturation in certain stages of development. But a role was observed in ephrin B2 signalling in rat neurons (84). However experiments performed with Pag1 knockout mice showed that Pag1 is dispensable for immunoreceptor signalling. (85-87). We found hypoxic up-regulation of PAG1 in immune cell derived cancer cell lines, such as HL60 and Jurkat (see 3. 1). Based on the mentioned before findings that the loss of PAG1 can be compensated by another unknown transmembrane adaptor in immune cells, we did not follow up these results (85-87). However it would be still interesting to investigate the potential phenotype of Pag1 knockout in mice exposed to hypoxia.

With the creation of our stable PAG1 knockdowns in two different cell lines, HeLa and MDA-MB-231, we aimed to investigate the potential role of the protein in Src signalling and in cancer. Analysis of the Src Tyr 419 phosphorylation status in these two cell lines, comparing shmock cells with shPAG1 cells, we found a PAG1 dependent regulation. Unfortunately this result was not reproducible, so we concluded that the activation of Src and the regulation in hypoxia is PAG1 independent. Using our knockdown cell models for functional investigations we observed two different outcomes, dependent on the cell line investigated. In HeLa cells, displaying a high hypoxic PAG1 induction and low basal levels, we saw minor or no differences in the PAG1 knockdown compared to the control cells. In this cell line we also observed the hypoxic up-regulation of Caveolin-1 (see 4.3.). This observation led to the conclusion, that the loss of PAG1 is compensated at least in hypoxia by Caveolin-1. This protein

was also found to be regulated by an HRE and this gene in a HIF target gene (25). A following up study revealed that Caveolin -1 is a Maz-HIF-2α dependent target in HCT 116 cells. The *in vivo* work in this study revealed an essential function of hypoxia regulated Cav-1 leading to disruption of intestinal tight junctions and increased barrier permeability in mice (88). Furthermore, another study by Wang and co-workers showing the first time the HIF-dependent regulation of Cav-1 in 786-O cells and more important by Cav-1 knockdown in HeLa cells, the same cell model as we used in our PAG1 study, revealed a ligand-independent cell migration and cell invasion (89). *In vivo* studies using Cav-1 knockout mice exposed to chronic hypoxia showed a severe phenotype by the induction of right heart failure (90). These results together with our findings suggest at least for the HeLa cell line model, Cav-1 rather than PAG1 is the major transmembrane adaptor implicated in cell signalling processes.

Interestingly we could find a striking difference in our second PAG1 knockdown model cell line, MDA-MB-231 showing high basal levels of PAG1 expression and low hypoxic induction (see 3. and 4.3.). We could also not find a role in Src regulation. But comparing shPAG1 and shmock cells we found in our functional assays a reduced proliferation rate under prolonged hypoxia. In addition in the aforementioned cells we could observe the complete abrogation of the ability of these cells to grow anchorage-independent in a PAG1 dependent manner, regardless of the oxygen conditions. These results were independently observed by another study using cell lines with the same PAG1 expression pattern. 786-O cells also exhibit high basal PAG1 expression levels. Knockdown experiments of PAG1 and functional assays of these cells showed the same phenotype in anchorage-independent growth. Molecular dissection of the loss of PAG1 in cell signalling processes revealed a role of PAG1 in RhoA activation. Furthermore a functional link of PAG1's PDZ to cytoskeletal stress fiber formation was shown (91). These findings in cell lines showing the same PAG1 expression level, could strengthen the role of PAG1 as proto-oncogene, at least in these cells. Further investigations on other possible PAG1 functions besides its role in Src regulation, like its influence in other Src family kinases activity or its direct role in other cellular signalling processes are needed. Thus we would like to encourage further research on this topic, which can lead to the finding of the still missing clear function for this ubiquitously expressed, widely hypoxia regulated gene.

5.7. References

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6. Contributions to publications and manuscripts

Alexandra Schörg, Sara Santambrogio, James Platt, Johannes Schödel, Maja T. Lindenmeyer, Clemens D. Cohen, Katrin Schrödter, David R. Mole, Roland H. Wenger and David Hoogewijs

"Destruction of a distal hypoxia response element abolishes *trans*-activation of the *PAG1* gene mediated by HIF-independent chromatin looping"

Nucleic Acid Research, submitted

All figures except figure 3C-E, figure 6A-C, supplementary figure 1, supplementary figure 2, supplementary figure 3 and supplementary figure 5.

Danielle L. P. Brooks, Luciana P. Schwab, David Hoogewijs, Alexandra Schörg, Lauren Gotwald, Roland H. Wenger and Tiffany N. Seagroves

ITGA6 (CD49f) is directly regulated by the Hypoxia-Inducible Factors (HIFs) and enriches for cancer stem cell activity and invasion potential in metastatic breast cancer models

PLoS One, in preparation

Creation of the shHIF-1α/2α double, single and control knockdowns

Tarah M Regan Anderson, Danielle L Peacock, Andrea R Daniel, Gregory K Hubbard, Kristopher A Lofgren, Brian J Girard, Alexandra Schörg, David Hoogwijs, Roland H. Wenger, Tiffany N. Seagroves

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Creation of the shHIF-1α/2α double, single and control knockdowns

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“ITGA6 (CD49f) is directly regulated by the Hypoxia-Inducible Factors (HIFs) and enriches for cancer stem cell activity and invasion potential in metastatic breast cancer models” *PLoS One (in preparation)*

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